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Canine Lipoprotein Separation and Characterization of the Canine Lipidome in Healthy and Prednisolone Treated Beagles

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Contents

Abstract

Changes in the lipid metabolism are known to mediate several diseases (e.g. atherosclerosis, coronary artery disease). The potential of lipidomics is increasingly recognized as clinical tool to assess pathomechanisms and biomarkers for several of these diseases.

The purpose of this study was first, to evaluate canine lipoprotein separation and second, to characterize the canine lipidome using an untargeted lipidomic approach.

After ultracentrifugation or precipitation of plasma and serum, fractions were analyzed by SDS–PAGES, indicating associated apoproteins. No accurate separation of the different lipoproteins was possible.

Lipidome analysis revealed a total amount of 561 different lipids from eight of eight categories in plasma and serum; 246 of them were identified in both matrices. The relative mean abundance was different in 11/246 lipids between the two matrices. Prednisolone treatment induced a significant increase of seven and a significant decrease of three lipids in plasma and a significant increase of two and a significant decrease of one lipid in the serum.

In conclusion, complete lipoprotein separation is still not possible in dogs, presumably due to their overlapping densities. Untargeted lipidomic analysis revealed marginal differences between lipids found in both, serum and plasma. Prednisolone treatment induced significant changes in the plasma lipidome, which should be evaluated in further studies.

Zusammenfassung

Veränderungen im Lipidmetabolismus spielen bei verschiedenen Erkrankungen eine Rolle (z.B. Atherosklerose, Herzkranzgefässerkrankungen). Untersuchungen des gesamten Lipidoms gewinnen daher zunehmend an Bedeutung, da damit die Pathomechanismen dieser Erkrankungen untersucht und neue Biomarker erforscht werden können.

Das Ziel dieser Studie war es, die Lipoproteine beim Hund zu separieren und das canine Lipidom mittels einer ungerichteten Lipidomanalyse zu untersuchen.

Nach Ultrazentrifugation oder Präzipitation von Plasma und Serum, wurden alle gewonnenen Fraktionen mit SDS–PAGES auf zugehörige Apoproteine untersucht. Eine exakte Auftrennung der Lipoproteine war nicht möglich. Bei der Lipidomanalyse wurden 561 Lipide aus acht von acht Lipidkategorien nachgewiesen. In beiden Materialien wurden 246 Lipide identifiziert, wovon bei elf ein signifikanter Unterschied in der mittleren, relativen Menge zwischen den zwei Medien bestand. Die Prednisolonbehandlung führte im Plasma zu einem signifikanten Anstieg von sieben, im Serum von zwei Lipiden und im Plasma zu einem signifikanten Abfall von drei, im Serum von einem Lipid.

Eine exakte Auftrennung der Lipoproteine beim Hund war, vermutlich wegen Dichteüberlappungen, nicht möglich. Die ungerichtete Lipidomanalyse ergab lediglich geringfügige Unterschiede zwischen Plasma und Serum. Die Prednisolonbehandlung führte zu signifikanten Veränderungen einiger Lipide, die in weiteren Studien genauer untersucht werden sollten.

Abbreviations

apo	Apoprotein
BHT	2,6–di–tert–butyl–4–methylphenol / Antioxidant
CA	Calcification cores
CE	Cholesteryl ester
Cer	Ceramide
CETP	Cholesteryl ester transfer protein
CHOL	Cholesterol
CM	Chylomicron
COX	Cyclooxygenase
CVD	Cardiovascular disease
d	Density
DG	Diacylglycerol
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acyl
FC	Foam cells
fdr	False discovery rate
FFA	Free fatty acids
FPLC	Fast protein liquid chromatography
GC	Glucocorticoid
GL	Glycerolipid
GP	Glycerophospholipid
HAC	Hyperadrenocorticism
HCH	Hypercholesterolemia
HDL	High density lipoprotein
HL	Hepatic lipase
HTG	Hypertriglyceridemia
IDL	Intermediate density lipoprotein
LC	Liquid chromatography
LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LP	Lipoprotein
Lp (a)	Lipoprotein (a)
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
m/z	mass to charge ratio
MAC	Macrophages
MG	Monoacylglycerol
MLP	Modified lipoproteins
MON	Monocytes
MS/MS	Tandem mass spectrometry
MTBE	Methyl *tert*–butyl ether
PA	Glycerophosphatidic acid
PC	Glycerophosphocholine
PE	Glycerophosphoethanolamine
PG	Glycerophosphoglycerol
PI	Glycerophosphoinositol
PK	Polyketide
PL	Platelets
PR	Prenol lipid
PS	Glycerophosphoserine
PUFA	Polyunsaturated fatty acid
R_f	Relative migration distance
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SL	Saccharolipid
SM	Sphingomyelin
SMC	Smooth muscle cells
SP	Sphingolipid
ST	Sterol lipid
TG	Triglyceride / Triacylglycerol
TLY	T–lymphocytes
VLDL	Very low density lipoprotein

Chapter 1

Introduction

1.1 Canine Lipoproteins and Lipid Metabolism

Lipids, including triglycerides and cholesterols, are essential for a normal physiological function of the organism. Triglycerides are the most common and efficient source of chemical energy, while cholesterol, the main sterol in animal tissue, is a structural component of cell membranes and an essential precursor in steroid hormone and bile acid synthesis (Camus et al., 2011; Xenoulis and Steiner, 2010). Because of their water–insolubility, transportation of lipids through plasma to their sites of utilization is provided by lipoproteins (LPs). LPs are composed of a surface coat, containing phospholipids, free cholesterol and apoproteins (apos), surrounding a hydrophobic lipid center containing triglycerides (TGs) and cholesteryl esters (CEs), Fig. 1.1. The apoproteins, also called apolipoproteins, are specific proteins which direct the lipoproteins to their sites of metabolism and act as cofactors in enzymatic reactions (Camus et al., 2011; Bauer, 2004).

1.1.1 Canine Lipoproteins

There are four discrete populations of canine lipoproteins, classified on the basis of their size, density, lipid and apoprotein content, and electrophoretic mobility, with lipoproteins being larger and less dense if the fat to protein ratio is increased. Each lipoprotein species has specific functions. The classes recognized in the dog are: chylomicrons (CMs), very low density lipoproteins (VLDLs), low density lipoproteins (LDLs) and high density lipoproteins (HDLs), which is a heterogeneous group of lipoproteins, displaying several subfractions such as HDL_1, HDL_2 and HDL_3 as the most important ones (Camus et al., 2011).

CMs and VLDLs predominantly transport triglycerides, LDLs while HDLs predominantly transport cholesterol (Camus et al., 2011). As an exception, non–esterified free fatty acids form complexes with albumin in the circulation and do not require incorporation into lipoproteins for transportation (Johnson, 2005).

In contrast to humans, whose LDLs are the major lipoproteins and cholesterol carriers, the dog is a species with very few LDLs and predominantly HDLs. Hence, the majority of

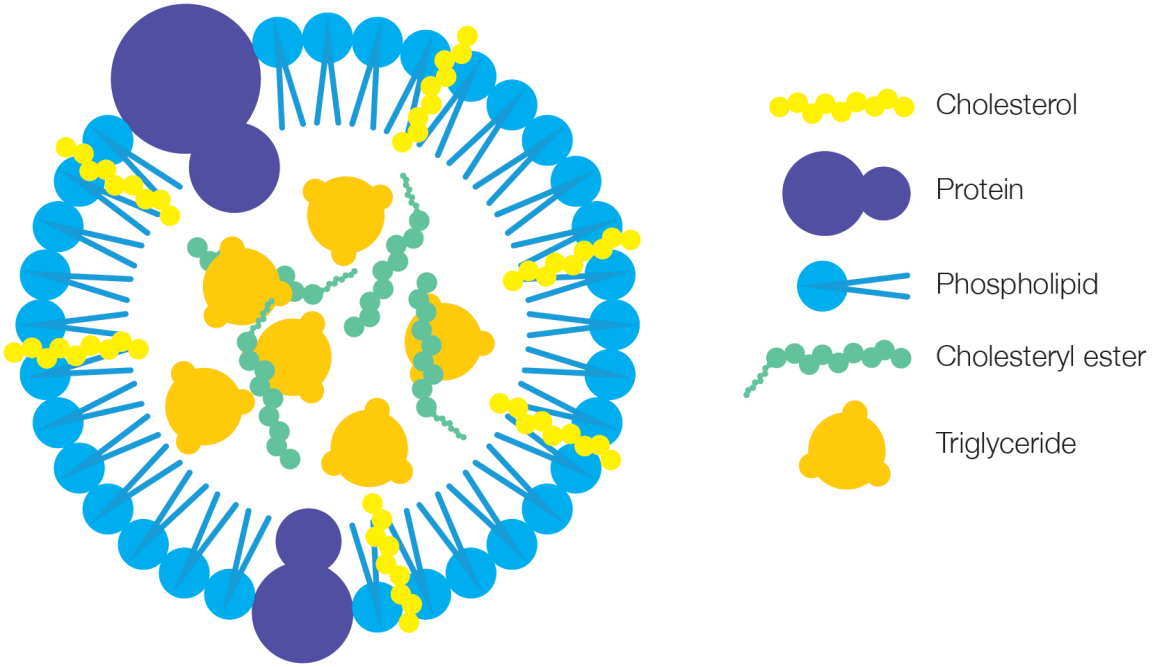


Figure 1.1: Lipoprotein model

circulating cholesterol is carried by HDLs as well, with HDL_2 being the main cholesterol carrier in dogs (Mahley and Weisgraber, 1974). Canine HDL and LDL contribute with nearly 87% and 11%, respectively, to the total cholesterol concentration of plasma in dogs (Maldonado et al., 2001). In addition to the higher percentage of HDL in canine blood, LDLs have a higher content of triglycerides than has been reported for the LDL class in humans (Mahley and Weisgraber, 1974).

There is no general consensus on the exact lipoprotein classes in dogs regarding size, density ranges and major apoproteins. The most popular canine lipoprotein studies are those of Mahley and Weisgraber (1974), Bauer (2004) and Xenoulis and Steiner (2010) as described in table 1.1.

CMs are similar to human CMs, consisting mainly of dietary triglycerides (TGs), 75–1200 nm in size, with a density of <0.96 g/mL and tending to be at the origin of electrophoretic mobility. Apoprotein (apo) B–48 functions as the major apoprotein for CMs (Mahley and Weisgraber, 1974).

VLDLs consist primarily of endogenous triglycerides, measure 30–80 nm in size, possess a density of 0.093–1.006 g/mL and a pre–β electrophoretic mobility. Their major apoproteins are B–48 and B–100, but apo E and C were found as minor apoproteins in the dog as well (Mahley and Weisgraber, 1974).

LDLs contain phospholipids and cholesteryl esters, are 18–25 nm in size, have a density of 1.019–1.087 g/mL, show β mobility on electrophoresis and have B–48 as well as B–100

Table 1.1: Characteristics of lipoproteins in dogs (Bauer, 2004; Xenoulis and Steiner, 2010).

Lipoprotein	Main Consistency	Size (nm)	Hydrated Density (g/mL)	Electro–phoretic Mobility	Major Apopro–teins
Chylomicrons	Dietary Triglycerides	75–1200	<0.96	Origin	B–48, (C)
VLDL	Endogenous Triglycerides	30–80	0.093–1.006	Pre–β	B–100, B–48, E, C
LDL	Phospholipids, Cholesteryl esters	18–25	1.019–1087	β	B–100, B–48
HDL_1	Phospholipids, Cholesteryl esters	10–35	1.025–1.100	α_2	E, A, C
HDL_2	Phospholipids	9–12	1.063–1.100	α_1	E, A, C
HDL_3	Phospholipids	5–9	1.100–1.210	α_1	A, C

as major apoproteins (Mahley and Weisgraber, 1974).

The last lipoprotein fraction, HDLs, is divided into three subfractions in dogs, whereas in humans up to 14 subfractions are known. Canine HDL_1 is a unique canine lipoprotein, unlike any of the commonly described lipoproteins in humans, containing phospholipids and nearly as much cholesterol as the LDL class. With 10–35 nm, they overlap in size with LDLs. Further they possess a density range of 1.025–1.100 g/mL, also overlapping with LDLs, and an α_2 electrophoretic mobility. Major apoproteins are apo E, A and C (Maldonado et al., 2002). The canine HDL_2 consists mainly of phospholipids but also carries most of the total plasma cholesterol, is 9–12 nm in size, has a density range of 1.063–1.100 g/mL, an α_1 mobility on electrophoresis and apo E, A and C as major apoproteins. Finally, the canine HDL_3 consists mainly of phospholipids as well, measures 5–9 nm in size and has also the highest density range with 1.100–1.210g/mL, an α_1 mobility on electrophoresis and apo A and C as major apoproteins (Mahley and Weisgraber, 1974). Several apoproteins can further be divided into subfractions, such as apo A–I, apo A–II and so on, similar to apo B–48 or apo B–100.

1.1.2 Canine Lipid Metabolism

Canine lipoproteins are formed either during exogenous or endogenous lipid metabolism; both mechanisms are graphically illustrated in figure 1.2.

Exogenous Lipid Metabolism

The exogenous lipid metabolism consists mainly of CMs, which are formed of triglycerides absorbed from the small intestine in combination with a small amount of cholesterol, phospholipids and apo B–48. Afterwards CMs enter the intestinal lacteal and are transferred through the lymphatic system into the blood circulation (Camus et al., 2011; Mooney

et al., 2004). Therefore, CMs may be detected in the blood if samples are taken postprandially. During transport through circulation, CMs acquire several other apoproteins, such as apo Cs and apo E from interaction with HDLs. (Bauer, 2004) Apo C–II acts as a co–factor to activate the enzyme lipoprotein lipase (LPL), which is attached to tissue endothelium such as the capillary beds of adipose and skeletal muscle tissue. LPL hydrolyzes TGs in the core of CMs into free fatty acids (FFA), mono– and diglycerides, and glycerol. FFAs become re–esterified within the tissue into TGs for storage, which are the main energy source for cells (Camus et al., 2011; Xenoulis and Steiner, 2010). After activation of LPL, the remaining chylomicron remnants are relatively deficient in TGs and enriched in cholesteryl ester (Mooney et al., 2004). CM remnants return their apo C–II to HDLs and are then removed from circulation by uptake into the liver through their remaining apo E (Bauer, 2004; Xenoulis and Steiner, 2010).

The activity of LPL can be modified not only by apoproteins, but also by a variety of hormones, including insulin, glucagon and catecholamines. Hence, the activity of LPL of the adipose tissue correlates positively with plasma insulin concentrations, ensuring delivery of fatty acids to the adipose tissue during the postprandial period (Camus et al., 2011).

Endogenous Lipid Metabolism

The endogenous lipid metabolism involves VLDLs, LDLs and HDLs. VLDLs and HDLs are synthesized continuously in the liver, with secretion being influenced by nutritional and metabolic changes, whereas LDLs are formed by transformation of VLDLs (Camus et al., 2011).

VLDLs are large macromolecules, although smaller than CMs, containing predominantly triglycerides (TGs) and in lesser amounts cholesterol, cholesteryl ester (CEs), phospholipids and apo B–100 as well as apo B–48 (Bauer, 2004). Most of the parts used for formation of VLDLs in the liver are either synthesized by the liver itself or redistributed after hepatic uptake of other lipoproteins (Mooney et al., 2004).

Upon entering the circulation, the metabolism of VLDLs resembles that of CMs. They acquire apo C and apo E from HDLs, with apo C–II activating the LPL, starting hydrolyzation of TGs identically to the metabolism of CMs (Xenoulis and Steiner, 2010). The resulting remnants of VLDLs are then also relatively deficient in TGs and enriched in CEs and can be removed from the circulation by hepatic uptake or they may be further modified to LDLs by hepatic lipase (HL), an enzyme bound to the hepatic endothelium (Mooney et al., 2004).

LDLs contain mainly cholesteryl esters (CEs), phospholipids and apo B–100, circulate in the blood and bind to specific receptors in order to deliver cholesterol to the peripheral tissues for further usage, such as synthesis of steroid hormones and cell membranes (Bauer, 1996).

In so–called reverse cholesterol transport, HDLs are responsible for the transport of cholesterol from peripheral tissues back to the liver. HDLs are primarily synthesized in the liver and intestines in the form of dense disc–shaped particles, so–called HDL_3, containing mainly phospholipids and apo A–I (Camus et al., 2011). These HDL_3 particles

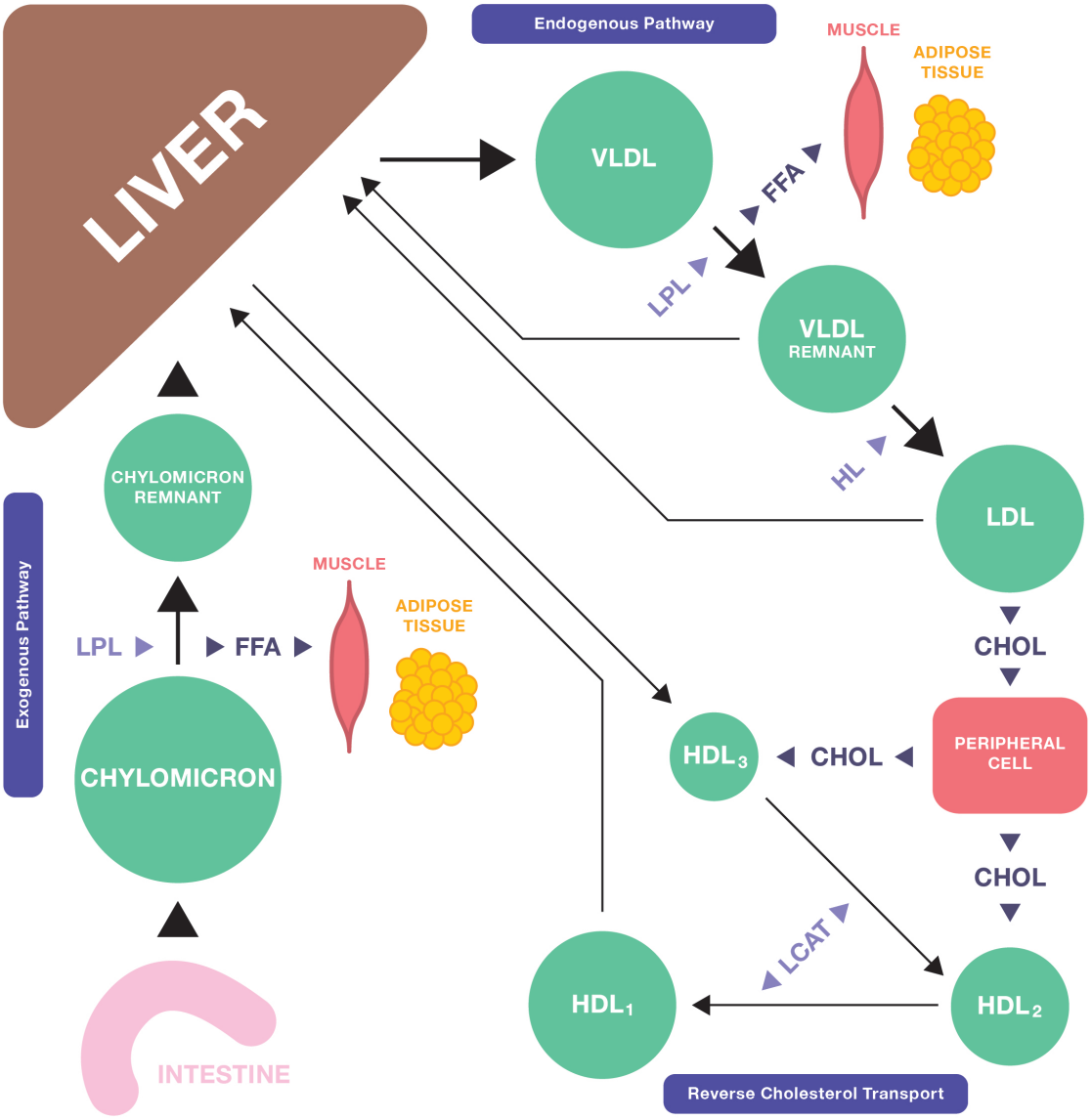


Figure 1.2: Canine lipid metabolism. LPL = lipoprotein lipase; FFA = free fatty acids; VLDL = very low density lipoproteins; HL = hepatic lipase; LDL = low density lipoproteins; CHOL = cholesterol; HDL = high density lipoproteins, LCAT = lecithin cholesterol acyltransferase.

draw cholesterol from peripheral tissue onto their surface, where free cholesterol is then continuously esterified into cholesteryl ester (CE) by the action of lecithin cholesterol acyltransferase (LCAT), activated by apo A–I (Mooney et al., 2004; Xenoulis and Steiner, 2010; Bauer, 2004). CEs move consecutively into the core of HDL_3, resulting in the formation of larger HDL_2 particles, allowing even more free cholesterol to be absorbed onto their surface. The final particles formed through the drawing of cholesterol and activity of LCAT are HDL_1, which are unique cholesteryl ester–rich particles. Finally,

CEs in the core of HDL_1 or HDL_2 are not transferred to other lipoproteins, but are directed to the liver for disposal or reuse, representing the last step of reverse cholesterol transport. Additionally, TGs of HDLs can be hydrolyzed in the liver, resulting in the conversion of HDL_2 back into HDL_3, the latter recirculating to the periphery, restarting the reverse cholesterol transport (Bauer, 2004; Li et al., 2014).

1.1.3 Difference of Canine and Human Lipid Metabolism

Although humane exogenous lipid metabolism functions similarly to that in dogs, some differences occur in the endogenous pathway.

Major Cholesterol Carrier

In humans, LDL is the main cholesterol carrier and is considered to be the major participating lipoprotein in the development of atherosclerosis (Camus et al., 2011). This is in contrast to dogs, where HDLs have a higher concentration within the blood than LDLs, rendering the reverse cholesterol transport more efficient in general by allowing more free cholesterol from peripheral tissue to be taken up into HDLs. This feature may be involved in the natural resistance to developing atherosclerosis in some mammals with enhanced HDL concentrations in the blood, such as the dog, since enhancement of cholesterol efflux and reverse cholesterol transport capacity is potentially antiatherogenic (Maldonado et al., 2002).

Intermediate Density Lipoprotein (IDL)

In humans, a further lipoprotein class has been described, the so–called intermediate density lipoproteins (IDLs). They might exist in dogs, as there is a gap between canine LDL and HDL density ranges, but they have only been described rarely. Further differences regarding canine and human lipoproteins and their composition are shown in table 1.2.

Reverse Cholesterol Transport

HDL_1, as described above in the canine endogenous lipid metabolism, is only known in mammals lacking a certain protein called cholesteryl ester transfer protein (CETP). In humans, CETP allows CEs to be transferred from HDL_2 back to apo–B containing lipoproteins such as LDLs or VLDLs in exchange for triglycerides (Johnson, 2005). This prohibits the formation of HDL_1 particles and furthermore enhances the percentage of LDL in the blood, allowing it to be the main cholesterol carrier in humans (Mooney et al., 2004).

The clearance of cholesteryl ester through HDL_1 in the dog, due to the absence or very low amount of CETP, is therefore considered to contribute to the cardioprotective role of HDL. Another presumed protective role of HDL is a higher resistance to oxidation than LDL (Warnick et al., 2001; Tsutsumi et al., 2001).

Table 1.2: Comparison of lipoproteins in dogs and humans. Table is composed of three different sources: Bauer (2004), Xenoulis and Steiner (2010) and Wasan et al. (2001).

Lipoprotein	Species	Size (nm)	Hydrated Density (g/mL)	Electro–phoretic Mobility	Major Apoproteins
Chylomicrons	Dogs	75–1200	<0.96	Origin	B–48, C
	Humans	75–1200	<0.95	Origin	B–48, A, C, E
VLDL	Dogs	30–80	0.093–1.006	Pre–β	B–100, B–48, E, C
	Humans	30–80	0.95–1.006	Pre–β	B–100, E, C
IDL	Humans	25–35	1.006–1.019		B–100, C, E
LDL	Dogs	18–25	1.019–1087	β	B–100, B–48
	Humans	18–25	1.019–1063	β	B–100
Lp (a)	Humans				
HDL_1	Dogs	10–35	1.025–1.100	α_2	E, A, C
HDL_2	Dogs	9–12	1.063–1.100	α_1	E, A, C
	Humans	9–12	1.063–1.125	α_1	A, E, C
HDL_3	Dogs	5–9	1.100–1.210	α_1	A, C
	Humans	5–9	1.125–1.210	α_1	A, E, C

Lipoprotein a

Lipoprotein (a), Lp (a), is a specific lipoprotein not fully described in dogs and of unknown physiological function. There are occasional reports of an unusual lipoprotein in the canine plasma, presumably Lp (a), occurring together with marked hypercholesterolemia and hypothyroidism (Camus et al., 2011). Human Lp (a) is believed to be an LDL particle containing additional apo (a) next to apo B–100. Hence it is not known if Lp (a) acts independently from LDL regarding the development of coronary vascular diseases, although studies have suggested that increased levels of Lp(a) in the plasma have been correlated positively with increased risk of coronary artery disease (Kronenberg, 2014; Danesh et al., 2000). Although pro–inflammatory oxidized phospholipids have been strongly associated with Lp (a) in human plasma, the underlying mechanisms by which Lp (a) contributes to the pathogenesis of atherosclerosis are not well understood (Tsimikas et al., 2004).

Apoproteins

Because of the large number of studies performed on human lipoproteins, more apoproteins and related metabolic functions are known in humans compared to canine lipoproteins (Table 1.3).

The majority of apoproteins have been found on HDLs, as they not only possess apo A–I and apo A–II as major apoproteins, but also minute amounts of apo C–I, apo C–II, apo C–III, apo E, apo A–IV and apo D. The sole apoprotein not found on HDL is apo B,

Table 1.3: Classification and properties of major human apoproteins with metabolic functions being mostly similar in humans and dogs (Jonas, 2002; Xenoulis and Steiner, 2010; Breslow, 1989). CM = chylomicron, VLDL = very low density lipoprotein, LDL = low density lipoprotein, IDL = intermediate density lipoprotein, HDL = high density lipoprotein, LCAT = lecithin cholesterol acyltransferase, CHD = coronary heart disease, LP = lipoprotein, LPL = lipoprotein lipase, TG = triglyceride, CE = cholesteryl ester.

Apolipoportein	Molecular Weight (kDa)	Lipoproteins	Major Metabolic Function
Apo A–I	28–29	HDL, CM	Activates LCAT; high levels associated with reduced risk of CHD
Apo A–II	17–18	HDL, CM	Enhances hepatic lipase activity
Apo A–IV	44–46	HDL, CM	Present in fat enriched LPs
Apo B–48	241–264	CM (plus VLDL & LDL in Dogs)	Secretion of CMs into lacteals; enhances hepatic uptake
Apo B–100	513–540	LDL, VLDL, IDL	Secretion of VLDLs from liver; hepatic uptake; high levels associated with increased risk of CHD
Apo C–I	6–7	CM, VLDL, IDL, HDL	Involved in activation of LPL
Apo C–II	8–9	CM, VLDL, IDL, HDL	Activation of LPL; deficiency results in accumulation of CMs and TGs
Apo C–III	8–9	CM, VLDL, IDL, HDL	Inhibition of LPL
Apo D	33	HDL	Closely associated with LCAT
Apo E	34–35	CM, VLDL, IDL, HDL	Uptake of CM remnants; binding to LDL–receptor; may inhibit development of atherosclerosis
Apo (a)	250–800	Lp (a)	Unknown

which is restricted to CMs, VLDLs, IDLs and LDLs (Warnick et al., 2001).

Regarding the subfractions of apo B, dogs possess apo B–48 not just on CMs, as in humans, but also on VLDLs and LDLs as well (Bauer, 1996). The inclusion of apo B–48 into VLDLs and LDLs in dogs may further explain their low concentrations of apo B–containing lipoproteins in the blood next to the occurrence of HDL_1, because of the possible rapid recognition and hepatic–receptor binding of apo B–48 together with apo B–100. Hence, more efficient clearance of particles containing both apoproteins may occur in the dog (Bauer, 1996).

1.1.4 Canine Dyslipidemia

As a general term, dyslipidemia involves all kinds of disturbances with lipids and lipoproteins (Xenoulis and Steiner, 2015). Hyperlipidemia is more specific and describes an in-

creased concentration of lipids in the blood, including cholesterol (hypercholesterolemia) or triglycerides (hypertriglyceridemia) or both (Jericó et al., 2009; Xenoulis and Steiner, 2015). In humans, hyperlipidemia, especially enhancement of LDLs, has long been known as one of the main risk factors for the development of atherosclerosis (Liu et al., 1986). The importance of specific forms of hyperlipidemia in dogs has come up only recently, as it was considered a relatively benign condition before (Xenoulis and Steiner, 2015).

A characteristic lipoprotein profile for healthy dogs is represented by low concentrations of VLDL and LDL and high concentrations of HDL (Mahley and Weisgraber, 1974). Therefore it was suggested that the higher concentration of HDL, supposedly due to the absence or minimal amount of CETP in dogs, decreases the risk of developing atherosclerosis (Jericó et al., 2009).

Nevertheless, hyperlipidemia in dogs has also been associated with several complications such as pancreatitis, liver disease, ocular disease, seizure and atherosclerosis (Xenoulis and Steiner, 2015).

It is important here to differentiate between pathological and physiological hyperlipidemia. The latter represents the most common form and occurs in non–fasted dogs through accumulation of chylomicrons (Johnson, 2005). Accordingly, any determination of blood lipid concentrations should always follow a fast of at least 12 hours.

Regarding pathological lipid disorders, primary hyperlipidemia may arise as a result of a defect in lipoprotein metabolism or, secondary hyperlipidemia, as a consequence of an underlying systemic disease (Camus et al., 2011; Jericó et al., 2009; Xenoulis and Steiner, 2015).

Table 1.4: Primary and secondary lipid disorders in the dog. Slightly modified and shortened table by author, original by Xenoulis and Steiner (2010, 2015). HTG = hypertriglyceridemia, HCH = hypercholesterolemia

Examples of Hyperlipidemia	Type of Lipid Abnormality	Increased Lipoproteins
Postprandial Hyperlipidemia	HTG (rarely HCH)	CM
High–fat Diets	HTG and/or HCH	HDL_1
Secondary Hyperlipidemia		
Diabetes mellitus	HTG (mainly) and/or HCH	VLDL
Hypothyroidism	HTG and/or HCH	HDL_1, VLDL, LDL
Hyperadrenocorticism	HTG and/or HCH	VLDL, LDL
Pancreatitis	HTG and/or HCH	VLDL, CM, LDL, HDL_1
Obesity	HTG and/or HCH	VLDL, LDL, HDL
Protein–losing Nephropathy	HCH	unknown
Cholestasis	HTG and/or HCH	LDL
Hepatic Insufficiency	HTG and/or HCH	unknown
Drugs (e.g. Glucocorticoids)	HTG and/or HCH	probably VLDL, LDL
Primary hyperlipidemia		
Miniature Schnauzer	HTG with or without HCH	VLDL, CM
Beagle	HTG and/or HCH	HDL_1, LDL
Shetland Sheepdog	HCH with or without HTG	HDL_1, LDL

Primary hyperlipidemia appears infrequently and is mostly associated with certain breeds and geographic regions, the most common being a hypertriglyceridemia mainly due to enhanced VLDLs, familial in breeds such as the Miniature Schnauzer, Shetland sheepdogs or Beagles (Watson and Barrie, 1993; Xenoulis and Steiner, 2015).

Secondary hyperlipidemia, the most common form of hyperlipidemia in dogs, is mainly associated with endocrine disorders such hypothyroidism, diabetes mellitus or hyperadrenocorticism (Xenoulis and Steiner, 2010, 2015). The main primary and secondary lipid disorders in dogs and respective lipoprotein abnormalities are summarized in table 1.4.

In spite of elevated LDL concentrations in the plasma of several of these secondary hyperlipidemias, the development of atherosclerosis still remains rare in dogs (Hess et al., 2003). It has been documented only in association with extremely high plasma cholesterol (>20 mmol/L or 750 mg/dL) and triglyceride concentrations (Kagawa et al., 1998). If occurring spontaneously, atherosclerosis has been reported mainly in dogs with hypothyroidism, a disease that often is associated with extreme levels of hypercholesterolemia (Liu et al., 1986; Hess et al., 2003). Further, atherosclerosis has also been reported to occur in dogs with diabetes mellitus, however this is less common and controlled studies to prove these associations have not been performed so far (Hess et al., 2003). Interestingly, dogs with hyperadrenocorticism do not seem to have enhanced risk of developing atherosclerosis (Hess et al., 2003).

Hyperadrenocorticism

Canine hyperadrenocorticism (HAC) can occur spontaneously or iatrogenically. If occurring spontaneously, HAC results most often from an increased secretion of adrenocorticotropic hormone (ACTH) due to an anterior pituitary neoplasia or less often from autonomous cortisol production from a functional adrenocortical neoplasia (Mooney et al., 2004). Either way, enhanced cortisol levels can be found within the blood as the normal feedback mechanism can no longer function (Mooney et al., 2004). In contrast, iatrogenic HAC is caused by administration of glucocorticoids.

Both naturally occurring and iatrogenic HAC have been associated with hyperlipidemia, especially increased concentrations of LDLs and VLDLs or increased total plasma cholesterol concentrations within these lipoproteins (Bauer, 2004; Jericó et al., 2009).

This increase may be explained by a reduced clearance rate of VLDL, caused by lower LPL activity and be the consequence of glucocorticoid antagonism on insulin action (Barrie et al., 1993a). In addition, HDL concentrations were lower compared to those of healthy dogs, probably due to a decreased activity of LPL as well, because HDL originates partly from surface components released from chylomicrons or VLDL during LPL activity (Shaw et al., 2005). Hypertriglyceridemia has also been shown in dogs with HAC. But both cholesterol and triglyceride concentrations are only mildly to moderately increased (Johnson, 2005).

Glucocorticoids

The administration of glucocorticoids (GCs) in dogs can lead to hypertriglyceridemia, probably due to increased hepatic synthesis of triglycerides and increased secretion of VLDL, similar to naturally occurring HAC (Mooney et al., 2004). Generally, GCs have various effects on the organism, influencing carbohydrate, protein and fat metabolism as well as acting as anti–inflammatory agents. The effects of GCs on carbohydrate metabolism can be summarized as an increase in the gluconeogenesis rate, which leads to an increase in the blood glucose concentration, and this hyperglycemia, in turn, leads to a secondary hyperinsulinemia (Mooney et al., 2004). The enzymes activated by GCs in protein metabolism, enhancing blood amino acid concentrations through muscle catabolism, affect the lipid metabolism indirectly by increased conversion of amino acids to glucose and increased formation of plasma proteins in the liver (Mooney et al., 2004).

Further, GCs activate several enzymes involved in the fatty acid synthesis, promoting the secretion of lipoproteins (Wang et al., 1995). This is caused by increased body fat mass, lipolysis and lipid oxidation, which leads to peripheral insulin resistance, inhibiting the effect of insulin on stopping lipolysis and stimulating lipogenesis (Kaur, 2014). Finally, lipolysis results in the release of free fatty acids and glycerol from adipose tissue, stimulating an overproduction of VLDLs and consequently also enhanced LDL levels (Barrie et al., 1993a).

1.1.5 Atherosclerosis

Arteriosclerosis is a general term for thickening and hardening of arterial walls, whilst atherosclerosis is a specific form of arteriosclerosis defined as plaques building up inside arteries. The pathogenesis of this latter process is characterized by a chronic inflammation of the arterial walls, probably initiated by endothelial dysfunction, followed by structural alterations which in turn permit the accumulation of LDLs within the arterial walls (Kwon et al., 2008).

Risk Factors for Atherosclerosis

There are several classic risk factors for the development of atherosclerosis in humans, including so called first class and second class risk factors. First class risk factors are the following: hypertension, causing endothelial damage; hyperlipidemia with increased LDL or VLDL but reduced HDL concentrations; nicotine; diabetes mellitus; progressing age and gender (related to the protective effect of estrogen). Second class factors rely more on environmental factors such as high fat diets, obesity, stress or lack of physical exercise (Böcker et al., 2008).

In contrast to other factors, dyslipidemia is a prerequisite for the initiation and progression of about half of all arterial lesions (Simionescu and Sima, 2012) with increased lipoprotein (a) and small dense LDL concentrations. The latter are LDLs that are smaller after hydrolysis, mainly of triglycerides (Kullo et al., 2000; Lammert and Zeeb, 2014). Other studies have strongly associated mainly oxidized LDL with atherosclerosis (Ekroos et al., 2010). As they invoke inflammatory response in the artery wall, unleashing many

of the processes involved in atherosclerosis, detection of oxidized LDL may represent a good tool as a disease biomarker (Packard and Libby, 2008). Generally, inflammation has recently gained in importance, as it represents a key contributor to all stages of atherosclerosis, decreasing the importance of oxidized lipoproteins and further revealing the complexity of the process (Libby et al., 2009).

However in general, dyslipidemia including elevated small dense LDLs, total blood cholesterol levels and decreased HDL concentrations still seem to be the most potent risk factors for coronary heart disease (Grundy, 2011; Yin et al., 2012; Sniderman et al., 2001).

As small dense LDLs are supposed to increase the risk for atherosclerosis independently of LDL–cholesterol, this may explain some resistance to cholesterol lowering therapies. Small dense LDLs hold various pro–atherogenic features such as the ability to penetrate the subintimal space, greater susceptibility to oxidation (Warnick et al., 2006), increased binding to the arterial wall, increased toxicity to endothelial cells and activation of further atherogenic processes contributing to the formation of foam cells (Sniderman et al., 2001).

Stages of Atheroma Formation

The accumulation of atherogenic lipoproteins involves the initial influx of particles into the intima following endothelial injury, known as the "Response to Injury" hypothesis, and creating an atheroma, which is defined as hardening of the arteries. Although LDL is the main lipoprotein involved in the process, VLDL and IDL may also accumulate in arteries (Bauer, 2004). After lipoproteins are retained by the intima of the arterial walls, the formation of the atheroma continues, involving several stages illustrated in figure 1.3.

- Stage I: The formation of an atheroma can be summarized as a chronic inflammatory process, started by a dysfunction of the endothelial cells. This is caused by altered haemodynamic factors such as hypertension. Due to the following structural alterations, proteoglycans of endothelial cells are exposed to the blood. LDLs can bind to these proteoglycans through their apo B–100, permitting subendothelial accumulation of LDL particles in the intima (Weber and Noels, 2011).

- Stage II: After LDLs are trapped within the intima and if cholesterol is not brought back to the liver through reverse cholesterol transport by HDLs at this point, LDLs are susceptible to oxidative modifications by reactive oxygen species or enzymes, mostly released from inflammatory cells (Weber and Noels, 2011). Oxidative modification of LDLs involves lipid peroxidation, which in turn enhances recognition and uptake of LDLs by macrophages (Bauer, 2004). Recent studies also associated glycation of lipoproteins (bonding with a sugar molecule) as a process potentially contributing to atherosclerosis (Soran and Durrington, 2011) as glycated LDLs are more likely to be oxidized than non–glycated LDLs (Ravandi et al., 2000).

- Stage III: Oxidized LDLs induce further endothelial cell dysfunctions, expressed by the appearance of new cell adhesion molecules and chemotactic factors (Wick and Grundtman, 2011). Triggered by these attracted cells, a robust inflammatory reaction takes place in which blood monocytes, platelets, T–lymphocytes and dendritic

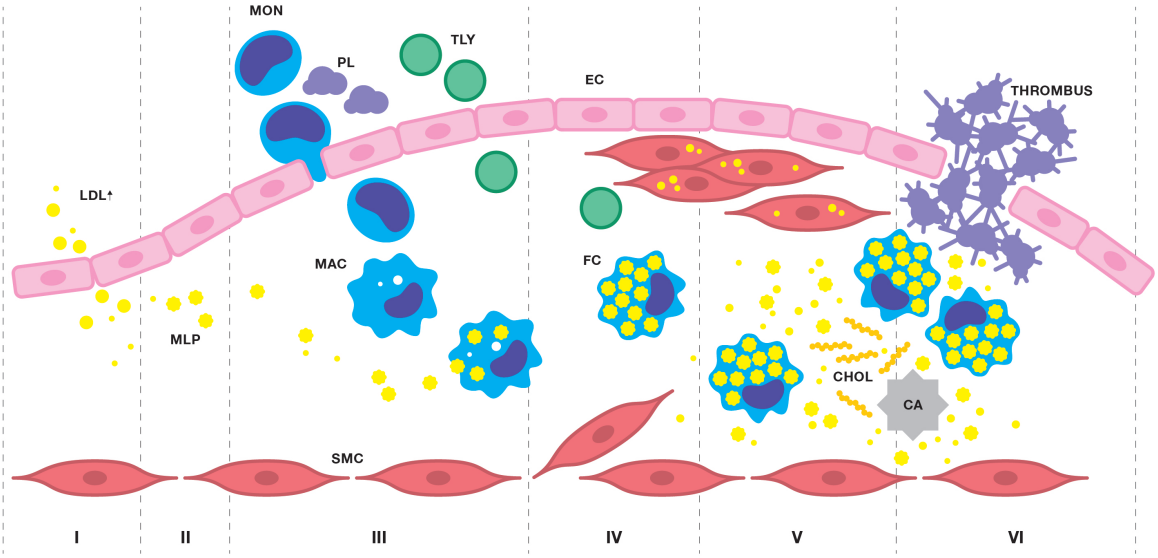


Figure 1.3: Diagrammatic Representation of stages I–VI of atheroma formation. Definitions: EC = endothelial cells; LDL = low density lipoproteins; MLP = modified lipoproteins; MON = monocytes; PL = platelets; TLY = T–lymphocytes; MAC = macrophages; FC = foam cells; SMC = smooth muscle cells; CHOL = cholesterol; CA = calcification cores; THROMBUS = thrombus. Figure adapted by author from Wick and Grundtman (2011).

cells adhere and enter the arterial intima (Wick and Grundtman, 2011). During this process, monocytes migrate to the sub–endothelial space where they differentiate into macrophages (Xiangdong et al., 2011). Once activated, macrophages express scavenger receptors, take up oxidized LDLs through these receptors and progressively turn into characteristic macrophage–derived foam cells (Wick and Grundtman, 2011). Under normal conditions, LDLs would be phagocytized through apo B–receptors, working with a feedback mechanism to stop cells from taking up too much cholesterol. This mechanism is lacking with scavenger receptors, allowing for the formation of large foam cells (Böcker et al., 2008). Ultimately, the accumulation of foam cells loaded with lipids and T–lymphocytes results in the formation of fatty streaks within the vessel walls, narrowing the arterial lumen (Xiangdong et al., 2011).

– Stage IV: Changes within the intima and influx of lymphocytes, interacting with macrophages, stimulates further production of IFN–γ. This enhances the ongoing inflammatory reaction and promotes immigration of smooth muscle cells from the media into the intima or sub–endothelial space. Immigrated smooth muscle cells may ingest modified lipoproteins, forming a fibrous cap or plaque (Ross, 1999; Böcker et al., 2008).

– Stage V: After formation of a fibroid plaque, more extracellular lipids, foam cells,

apoptotic cells and cholesterol crystals accumulate (Weber and Noels, 2011). Hence calcification centers develop. Moreover, free cholesterol is a potent inducer of the apoptosis of macrophage–derived foam cells, inducing a lipid rich necrotic core (Wick and Grundtman, 2011). Also, the smooth muscle cells and collagen which cover the fibrous cap are replaced by macrophages, thinning the cap and turning the fibroid plaque into a vulnerable structure whilst attracting more inflammatory cytokines (Wick and Grundtman, 2011).

– Stage VI: The last stage is characterized by the rupture of the vulnerable plaque, which allows blood components such as platelets to come into contact with exposed matrix, plaque lipids or tissue factors, resulting in the formation of a thrombus. Subsequent ischemic symptoms such as myocardial infarction may occur, depending on the location and magnitude of the process (Libby et al., 2009).

The Role of HDL

Conventionally, HDL is known to have several anti-atherogenic properties due to its role in the reverse cholesterol transport bringing cholesterol from peripheral tissue, including artery walls, back to the circulation and the liver. Therefore, high concentrations of HDL are traditionally believed to assist in lowering the risk of developing cardiovascular diseases. However, there is accumulating evidence that it is the molecular structure of HDL rather than circulating HDL–cholesterol levels which determine their functional properties (Yan et al., 2014).

Newer studies suggest that HDL acts as a reservoir of preformed hydroperoxides, which can be donated to VLDL by CETP via apo A–I and LCAT. Therefore both enzymes seem to have pro–oxidative activity during VLDL oxidation (McPherson et al., 2007). Despite LCAT acting as a pro–oxidant during VLDL oxidation, it also acts as an antoxidant during LDL oxidation. Thus HDL plays a double–edged role in the pathogenesis of atherosclerosis, attributable to both CETP and LCAT (McPherson et al., 2007). Further, the antioxidative activity of HDL depends on its subfraction, with small HDL_3 having significantly higher activity than large HDL_2, the antioxidative action being mostly associated with inactivation of LDL lipid hydroperoxides (Kontush et al., 2003).

HDL also seems to have anti–inflammatory and antithrombotic effects (Frohlich and Pritchard, 1989) and particularly HDL_3 has been linked to the down–regulation of cell adhesion molecules on vascular endothelial cells, thus blocking the progression of atherosclerosis. Additionally, it prevents to some extent the chemotaxis of monocytes and may inhibit platelet aggregation (McPherson et al., 2007).

However, the protective activities of HDL may vary between individuals in humans and under different disease states (Ekroos et al., 2010). In patients with coronary heart disease, several recent studies have provided evidence that HDL can also exert pro–inflammatory properties and loses its protective functions under certain circumstances such as enhanced serum amyloid A levels, which are present during inflammation. (Natarajan et al., 2010; Artl et al., 2000).

Further evidence for the double role of HDL is given by pharmacological interventions that increase HDL blood levels. This did not reduce the risk of cardiovascular disease,

pointing to the importance of determining HDL composition and its functional properties rather than only circulating HDL concentrations (Li et al., 2014).

Canine Atherosclerosis

It has been suggested that canine atherosclerosis differs from human atherosclerosis in that lipids are primarily present in the media and adventitia of atherosclerotic canine arteries (Jubb, 2007). Nevertheless, some similarities are observed, e.g. lipids are also deposited in the tunica intima, as described in humans and the infiltration of monocytes into subendothelial space can take place (Kagawa et al., 1998). Further, among all domestic animals, only dogs show a deposition of cholesterol and other lipids in the arteries in measurable amounts (Jubb, 2007).

As discussed in section 1.1.3, several reasons may contribute to the atherosclerotic resistance of dogs. First, the naturally high concentration of HDLs, being the major cholesterol carrier even in obese dogs (Jeusette et al., 2005), may lead to a more efficient reverse cholesterol transport from peripheral tissue known as an anti–atherogenic feature. Second, the low or absent activity of CETP in dogs contributes to their resistance, as more total cholesterol remains in HDLs, leading to the formation of unique HDL_1 particles (Maldonado et al., 2001).

1.2 Lipoprotein Separation

Separation of serum or plasma fractions can be useful for investigating lipoprotein changes in different diseases. Due to lipoproteins differing in size, density, electrical charge, lipid and apoprotein composition, there are different techniques for separating lipoprotein classes within the blood.

1.2.1 Sequential Preparative Ultracentrifugation

Ultracentrifugation depends on different densities of particles with two traditionally used approaches: analytical or preparative ultracentrifugation.

In analytical ultracentrifugation, a sample is centrifuged at high speed without splitting it up into different fractions, rendering this technique unsuitable for further analytical procedures where separated lipoprotein fractions are needed. (Xenoulis et al., 2013).

Using preparative ultracentrifugation, different salt solutions are added stepwise to separate the lipoprotein classes in multiple centrifugation steps, allowing sequential flotation of lipoprotein classes dependent on their density, size and shape (Mills et al., 1984). However, the disadvantage of this conventional ultracentrifugation procedure is the relatively long total spin time, where the complete fractionation of all lipoproteins may take up to five days.

Thus, a new technique called very fast ultracentrifugation was investigated, using rapid rotation speeds of up to 120'000 rpm and thereby lowering the total run time (Pietzsch et al., 1995). To further minimize processing artifacts, an ultracentrifugal sequential

flotation method was developed that used only small sample volumes and short centrifugation times at low temperatures but with high rotation speeds (Brousseau et al., 1993). Low temperatures are a prerequisite when processing lipoproteins, to minimize enzymatic transformation and autoxidation (Tong et al., 1998). However, in contrast to conventional protocols, when the temperature is lowered to 4°C, prolonged centrifugation time is required for lipoprotein separation due to the increased viscosity of the density gradients at the lower temperature (Mills et al., 1984).

1.2.2 Precipitation

There are two approaches to the precipitation of lipoproteins. Either precipitation is performed alone or triglyceride–rich lipoproteins such as VLDLs are eliminated before precipitation by a short ultracentrifugation step. The precipitation of lipoproteins without a previous ultracentrifugation step may cause incomplete precipitation due to the large amount of triglycerides. If HDL–cholesterol is measured afterwards, it may be slightly overestimated (Warnick et al., 1985).

Typically, a combination of polyanions (heparin or dextran sulfate) with divalent cations (magnesium or manganese) is used for precipitation of apo B–containing lipoproteins (Warnick et al., 1985). Both methods, the heparin manganese chloride and the dextran sulfate magnesium chloride, leave small amounts of apo B in the HDL supernatant, while some parts of HDL precipitate unplanned (Warnick et al., 2001). However, the heparin manganese chloride method is recommended by several authors and with this method, small dense LDL, known to be even more atherogenic than normal LDLs, may also precipitate (Hirano et al., 2005).

Precipitated lipoproteins in humans are commonly contaminated with other plasma proteins and their lipid composition may differ from the analogous fractions obtained by ultracentrifugation alone (Mills et al., 1984). A more precise technique for precipitation would be achieved using antibodies directed against apoproteins, called immunoprecipitation. Unfortunately, the availability of suitable antibodies can be challenging.

In the dog, a combined ultracentrifugation–precipitation (0.09M $MnCl_2$) technique has been evaluated, resulting in trace contaminations of the LDL precipitate with HDL apoproteins (apo A–I) analyzed on SDS–PAGE. Further, it was suggested that an apo E–rich subfraction of HDL in dogs may co–precipitate with LDL when a manganese chloride method is used (Barrie et al., 1993a).

1.2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) is a form of protein electrophoresis in which the movement of charged proteins within an electrical field results in their separation; it is illustrated in figure 1.4. Therefore it can be used to evaluate apoprotein distribution among lipoprotein fractions separated by the above–mentioned methods such as ultracentrifugation or precipitation.

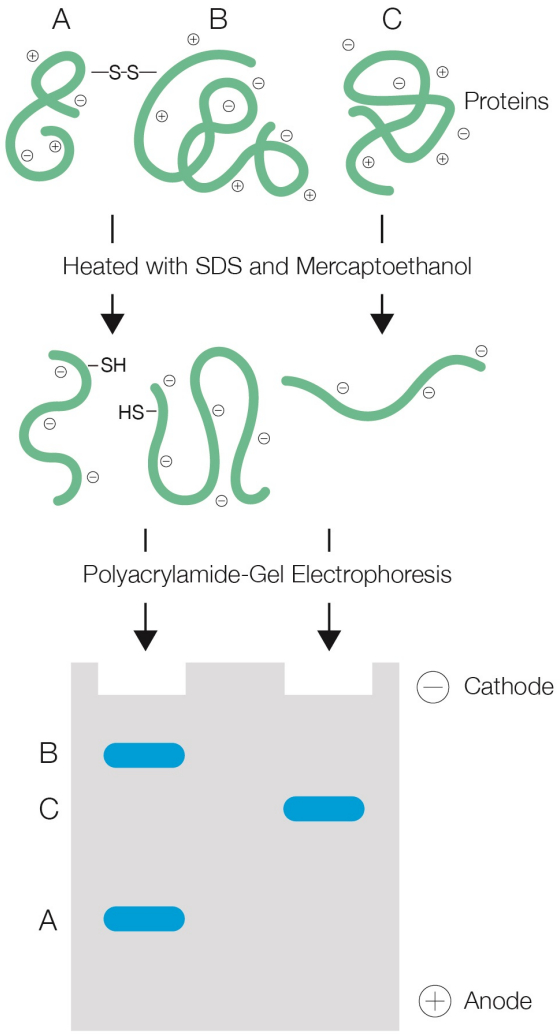


Figure 1.4: Mechanism of SDS–PAGE. Figure adapted by author from Raff et al. (2002).

Proteins can be negatively or positively charged, depending on their composition. Transferred into an electrical field, the proteins will migrate at a rate depending on their net charge, size and shape. Because of these different net charges, SDS–PAGE uses a powerful negatively charged detergent added to the protein solution called sodium dodecyl sulfate (SDS), which causes unfolding of proteins into extended polypeptide chains. In addition, β–mercapto–ethanol is added to the protein solution to break any disulfide bonds and separate all protein fragments from each other (Raff et al., 2002). This results in apoproteins released from other proteins or lipids becoming available to be studied alone in the different fractions of their assigned lipoproteins. Further, proteins of the same size will move at similar speed, because their native structure is completely unfolded and they possess the same amount of negative charge. Hence, large molecules are slower than small ones, resulting in a fractionation of proteins arranged by molecular weight with large

weight molecules at the beginning of the gel and small weight molecules further down the field (Raff et al., 2002).

Molecular weights of proteins detected on SDS–PAGEs can further be roughly estimated using the relative migration distance (R_f) of proteins; the migration rate of a protein on SDS–PAGE is inversely proportional to the logarithm of its molecular weight (Raff et al., 2002).

1.3 Lipidomics

By definition, lipidomics characterizes the composition of intact lipids in biological systems by identifying and quantifying intact molecular species of lipids (Ekroos et al., 2010; Postle, 2012).

Recently, there has been a dramatic increase in lipidomic research due to technical improvements such as the application of electrospray ionization to mass spectrometry (Postle, 2012). Lipidomics can be used not only for researching consecutive lipid changes during diseases, but also to map the entire spectrum of cellular lipids in biological systems, including metabolic pathways as well as lipid–lipid and lipid–protein interactions (Li et al., 2014).

There has already been an effort to identify the oxidation products of lipid species such as phospholipids, sterols and apoproteins formed in vivo and probably contributing to the development of atherosclerosis (Tong et al., 1998). In regard to dogs and their natural resistance to developing atherosclerotic plaques (Hess et al., 2003) lipidomic research on canine LDL, HDL and specific lipids and apoproteins seems promising. However, to our knowledge, the canine lipidome has not been characterized up to now.

1.3.1 Lipid Classification (Lipid Maps)

Lipids can be classified into eight major categories according to the Lipid Maps consortium (www.lipidmaps.org), where more than 500 distinct lipid species have been quantified so far from human samples, but many more have been identified (Postle, 2012). However, to define all lipid molecular species is challenging due to the extraordinary number of possible combinations. It has been recently postulated that the theoretical number of possible identifiable lipids in the human organism may approach 200'000 distinct molecular species, if all isoforms are included (Bou Khalil et al., 2010).

Further, lipids consist of a number of structurally and functionally distinct molecular species that extend from apolar (e.g. sterol esters) via neutral (e.g. triglycerides) to polar (e.g. phospholipids). Therefore it is challenging to carry out suitable identification techniques for each lipid and to integrate lipid data from different laboratories that have used different techniques (Hu et al., 2009).

Lipid Maps has gathered all this information and announced a lipid biology–approach system, whereby lipids can be classified according to their chemical structure: fatty acyls (FAs), glycerolipids (GLs), glycerophospholipids (GPs), sphingolipids (SPs), sterol lipids (STs), prenol lipids (PRs), saccharolipids (SLs) and polyketides (PKs).

The first group of lipids, fatty acyls (FAs), form the basic element of all lipids. They have either saturated or unsaturated straight hydrocarbon chains varying in lengths and amount of double bonds. Functionally, FAs are precursors of other lipid molecules, such as arachidonic acid, the precursor of eicosanoids (Hu et al., 2009).

Glycerophospholipids (GPs) represent the major group of lipids in the human organism and cover all sorts of mixtures of glycerol with a functional polar head group at the sn-3 position. Similar to glycerolipids, GPs are constituted of a glycerol backbone but with a phosphate esterified to one fo the glycerol hydroxyl groups (Fahy et al., 2005). They can be divided into subgroups based on these different polar head groups as follows: glycerophosphatidic acids (PAs), glycerophosphocholines (PCs), glycerophosphoethanolamines (PEs), glycerophosphoserines (PSs), glycerophosphoglycerols (PGs) and glycerophosphoinositols (PIs) (Hu et al., 2009).

Despite the diversity in their structure, all of these GPs are main components of cell membranes as parts of phospholipid bilayers and participate in cell signaling, membrane anchoring and substrate transport (Hu et al., 2009). In addition, some lipid molecular species (Lyso–PCs, PEs, PCs and PIs) have been found as potential biomarkers in various diseases such as pancreatic and ovarian cancer (Kolak et al., 2007).

Sterol lipids (STs), compromised of a four–ring core structure, consist of cholesterols and their derivatives, such as cholesteryl esters (CE). STs act as important components of membrane lipids, have regulatory functions related to cell signaling and contribute to the production of bile acids, mainly in the from of cholesterols. Steroid hormones, formed from STs, are separated into groups depending on their number of carbon atoms, for example C21 including progestagenes, glucocorticoids and mineralocorticoids (Tsai and O'Malley, 1994).

Glycerolipids (GLs), relatively simple lipids, mainly include monoacylglycerols (MGs), diacylglycerols (DGs) and triacylglycerols (TGs), synonymous with triglycerides. GLs consist of a glycerol backbone, varying only in the number of FAs esterified to the hydroxyl groups of this backbone. In nature, TGs are the main constituent of animal fats and therefore play an essential role in cellular energy storage. They have also been associated with the development of atherosclerosis (Goldberg, 1996; Hu et al., 2009).

Sphingolipids (SPs) together with glycerophospholipids (GPs) and cholesteryl esters (CEs) play an important role in the development of atherosclerosis, as they may alter lipoprotein functions. Ceramide (Cer), a main class of sphingolipids, affects this process through its role as second–messenger. Further, the concentration of sphingomyelin (SM), another class of sphingolipids, was positively correlated with the development of atherosclerosis in previous studies (Bismuth et al., 2008; Fahy et al., 2005).

1.3.2 Human Lipidome

The most detailed quantitative characterization of the human plasma lipidome was done by Quehenberger et al. in 2010, quantifying over 500 distinct lipid molecular species within the human plasma.

In that study, glycerolipids (GLs), mainly TGs, were found to be the most abundant lipids in human plasma, detected in CMs, VLDLs and IDLs. Next to TGs, cholesteryl

esters (CEs) were identified to be one of the most abundant lipid classes in human blood. Further, a large number of glycerophospholipids (GPs) were identified, with the majority of GPs being glycerophosphocholines (PCs) and glycerophosphoethanolamines (PEs).

In the group of sphingolipids (SPs), over 200 individual lipid species were assessed in which sphingomyelins (SMs) accounted for the largest fraction. Specific lipids such as ceramides (Cers) were mainly detected in VLDL and LDL fractions, whereas Lyso–PCs and ether–linked PCs were found in all lipoprotein fractions but were most abundant within HDL_2, HDL_3 and LDL. As this was a human study, no informations is available for HDL_1 (Quehenberger et al., 2010).

The observations of Quehenberger et al. were similar to those of other human lipidome studies, where major human lipoprotein components consisted of free cholesterol, chole–steryl esters (CEs), triglycerides (TGs) and phospholipids (PLs), particularly phosphatidyl-cholines (PCs) and sphingomyelins (SMs) (Wiesner et al., 2009). However, the abundance of different lipids varies between the studies because lipid profiles are affected by factors such as gender, age, race, current diet and the analytical approach used (Hyötyläinen and Orešič, 2015).

1.3.3 Lipidomics and Atherosclerosis

Lipidomics is an essential tool used to determine lipid molecular species as potential biomarkers in lipid–related diseases (Hu et al., 2009). As one of the first studies, atherosclerotic plaques were investigated, revealing that different (oxidized) lipids were enhanced exclusively within these plaques (Waddington et al., 2001).

Several lipids or lipid categories have been associated with atherosclerosis so far, for example oxidized phospholipids (ox PLs) (Berliner et al., 2009) with oxidized phosphatidyl-cholines specifically (Stübiger et al., 2012), sphingolipids (SPs) (Bismuth et al., 2008) and Lyso–PLs (Schmitz and Ruebsaamen, 2010). Further, cholesteryl esters, ceramides and lactosylceramide species were also associated with atherosclerotic plaques (Cheng et al., 2015), representing important lipid biomarkers for further investigations.

Regarding the antiatherogenic potential of HDL, lipidomic analyses are also important in characterizing HDL subfractions. The human HDL lipidome has been evaluated in various studies, e.g. revealing negatively charged PS and PA enriched in small, dense HDL_3, their content positively associated with anti–atherogenic functions (Berliner et al., 2009).

However, despite the great number of lipidomic studies performed in atherosclerotic research so far, we are still at the beginning of understanding lipid abnormalities in atherosclerosis. Most lipids identified and reported to be associated with atherosclerosis have not been investigated until now and their metabolic activity often remains unclear. Nevertheless, identification of these lipids would be an important step in moving forward.

1.3.4 Analytical Methods in Lipidomics

As lipidomic procedures consist of several different steps and every step has an effect on the measured lipid species, it is mandatory to consider several analytical factors.

Sample Matrices

Plasma and serum are known to be lipid–rich biofluids, containing many thousands of distinct lipid molecular species (Quehenberger et al., 2010). Proteins are the main class of interfering compounds in serum lipid analysis and their removal in advance of analysis is therefore important (Teo et al., 2015). Further, as plasma and serum differ in their composition, both matrices should be examined for their complete lipidome.

As no consensus on an optimal matrix for lipidomics in general has been reached, the type of matrix should always be chosen according to the targeted lipids (Ishikawa et al., 2014).

Storage and Handling

This is a critical point, as samples should always be stored immediately at −80°C or kept on ice during handling. Such conditions inhibit enzymatic activity and peroxidation as well as hydrolytic degradation of lipids. Hence, repeated freeze–thaw cycles should be avoided, which may lead to possible loss of metabolites such as PCs (Teo et al., 2015). Lysophospholipids (Lyso–PLs), one of the biomarkers for atherosclerosis, were found to be highly sensitive to higher temperatures. Even plasma samples kept at 4°C for 24h showed increased concentrations of Lyso–PCs, a subgroup of GPs (Yang et al., 2013).

Lipid Extraction

Generally, lipidomic analysis requires some form of lipid extraction. As lipids consist of hydrophobic and hydrophilic parts in varying amounts, the organic solvent needs to have a solubility specific to targeting the desired lipid. TGs and CEs are neutral lipids without any polar head groups and thus dissolve readily in nonpolar solvents such as hexane and also in moderately polar solvents such as chloroform, used in the traditional Folch lipid extraction (Hyötyläinen and Orešič, 2015; Folch et al., 1957). More polar lipids, such as phospholipids and sphingolipids are only slightly soluble in nonpolar solvents but have good solubility in polar solvents such as methanol. Hence, sample preparation always affects the detected metabolite profile and a single extraction method will therefore never represent all lipid subclasses adequately.

Currently there are two protocols that are used most commonly. One is a classic protocol based on a modified extraction using chloroform and methanol (2:1 v/v) invented by Folch et al. (1957). The other, newer protocol proposed by Matyash et al. (2008) uses methyl *tert*–butyl ether (MTBE), methanol and water (10:3:2.5 v/v/v). Results from Matyash et al. indicate that with the MTBE extraction protocol the recovery of most lipids is comparable to or in some lipid classes even more efficient than the traditional Folch method.

Internal Standards

Internal standard lipids can be added to the sample to correct differences in recoveries between lipids. However, even class–specific standards cannot fully account for the dif-

ferent extraction behaviors within lipid subclasses. For accurate quantification, targeted lipidomics with internal standards for each individual lipid is required (Cajka and Fiehn, 2014).

Liquid chromatography mass spectrometry (LC–MS) or Shotgun Lipidomics

Shotgun lipidomics and LC–MS based lipidomics are the two most widely used approaches (Ekroos et al., 2010).

Shotgun lipidomics is able to give a good overview of the lipids within a sample by detecting hundreds of molecular lipid species in non–separated lipid extracts. However, this often happens at the cost of less reliable lipid identification, as lipids are not separated before mass spectrometry, enhancing the rate of structural isomers. Isomers are different molecules but with the same molecular weight (Ekroos et al., 2010; Yang et al., 2015).

If already focused onto a specific lipid class or subclass, LC–MS based lipidomic analysis allows absolute quantification, often controlled by appropriate lipid standards (Ekroos et al., 2010). Further separation of isomers is partly achieved by previous LC (Watson, 2006). The drawback of this approach is the narrow time window when using LC, resulting in a limited number of identified lipids (Ekroos et al., 2010).

In both approaches, molecules usually undergo electrospray ionization (ESI), producing ions followed by the detection of mass–to–charge ratios for each charged particle by a mass analyzer. Together with characteristic fragmentation patterns resulting from tandem mass spectrometers, this allows a reliable identification of the lipid species (Ivanova et al., 2007; Carrasco-Pancorbo et al., 2009). However, the detection of lipid species with a very low abundance remains a major challenge regardless of the lipidomic approach (Wenk, 2010).

Data Processing and Lipid Identification

There are several tools available for data processing and identification with the core functions peak detection, filtering, artefact removal, alignment (for LC–MS data) and normalization. Common fragments (e.g.H^+, COO^-,...) possibly added to lipid molecules during analysis have to be supplemented to data for enhanced identification of lipids. Software tools have been developed for further identification of lipids using MS/MS in both positive and negative mode, adopting the lipid classification system introduced by Lipid Maps (Hyötyläinen and Orešič, 2015).

1.4 Objectives of the Study

Based on the information outlined above, two major objectives were addressed in the present study.

1. Firstly, to determine whether canine lipoproteins can be separated satisfactorily using ultracentrifugation or precipitation.

2. Secondly, to characterize the canine lipidome and to give an overview of the different lipid categories present in dogs. In the first part, we wanted to investigate a suitable matrix for the lipid extraction by comparing mean abundances of lipids in plasma and serum. In the second part, we wanted to evaluate the influence of short–term prednisolone treatment in healthy beagle dogs on the lipidome.

Chapter 2

Material and Methods

2.1 Lipoprotein Separation

Canine lipoprotein fractions were isolated from lithium heparin plasma or serum and were separated using two different techniques. A detailed description of each protocol is described in this section and an overview of the two protocols is depicted in figure 2.1.

Protocol A used ultracentrifugation at densities known to separate human lipoproteins and resulted in separation of the samples into five fractions (lipoproteins with density (d) <1.006 g/mL, d 1.006–1.019 g/mL, d 1.019–1.063 g/mL, d 1.063–1.210 g/mL and d >1.210 g/mL). The physiological arrangement of lipoproteins after ultracentrifugation is illustrated in figure 2.2.

Protocol B used heparin manganese chloride for precipitation and resulted in separation of the samples into two fractions (apo B–containing precipitate and supernatant).

Blood Samples

Lithium heparin and serum samples were collected from a healthy dog by puncture of jugular vein after 12 hours' fasting. Informed consent was obtained from the owner of the dog and its use was in accordance with the guidelines and directives established by the Animal Welfare Act of Switzerland (TVB NR 133/2013).

After separation of plasma or serum by centrifugation at 1500 $\times$ g for 10 minutes, samples were kept on ice at 4°C during analytical procedures or stored at −80°C. All samples were analyzed within 48 hours of collection.

Reagents

Sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), potassium bromide (KBr), manganese chloride solution (manganese(II) chloride tetrahydrate), tris hydrochloride (tris–HCl), sodium dodecyl sulfate (SDS), glycerol, bromophenol blue (BPB) and β–mercaptoethanol were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland); Precision Plus Protein^{TM} All Blue Marker was purchased from Bio–Rad, Switzer-

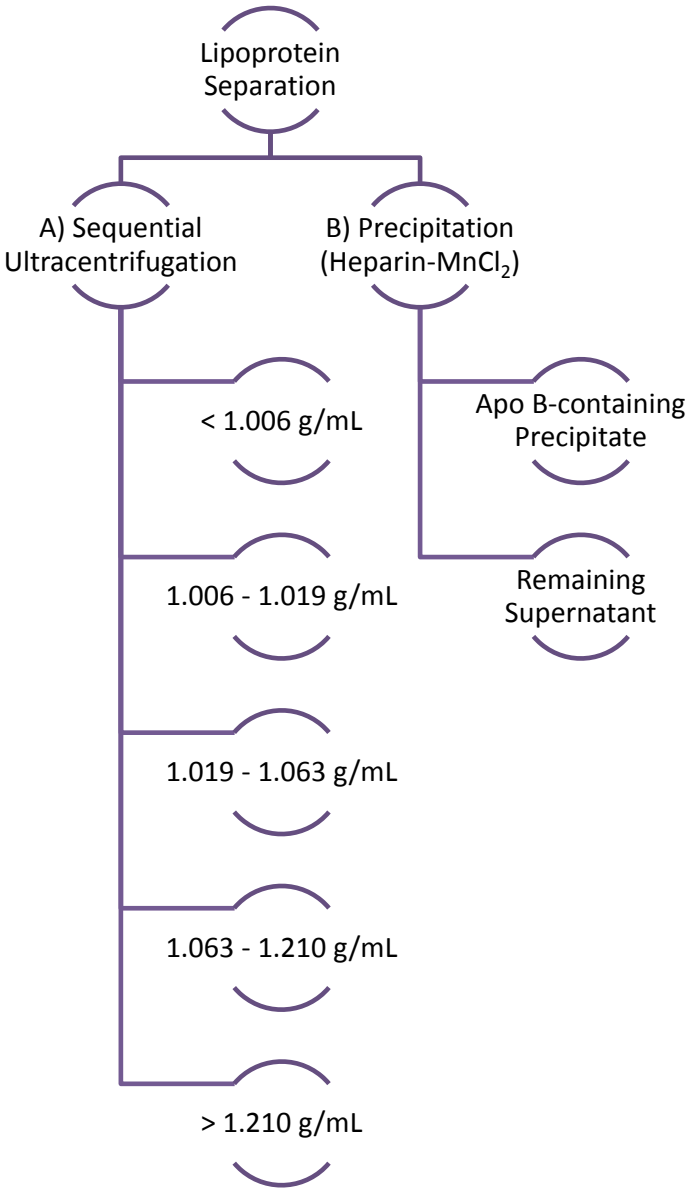


Figure 2.1: Different lipid separation techniques used in section 2.1.

land and heparin–sodium injection (5,000 I.U./mL) was purchased from B. Braun Medical AG (Sempach, Switzerland).

2.1.1 Protocol A: Ultracentrifugation

Canine lipoproteins were separated into five fractions from lithium heparin plasma or serum, using a protocol working strictly according to human lipoprotein densities and shorter run times, called protocol A, a modified protocol of Brousseau et al. (1993).

For ultracentrifugation, a benchtop ultracentrifuge (Beckman Optima TLX) with fixed angle rotor (TLA 120.2) operating at 120,000 rpm and maintained at 4°C was used with Quick–Seal® tubes (Beckman Coulter, Nyon, Switzerland).

All density solutions used for this protocol were made of potassium bromide (KBr) and distilled water, containing 1 mM EDTA and adjusted and verified by measurement on a digital density meter (Automatic Density Meter DS7800 from A. Krüss Optronic). Proportions were calculated according to a formula invented by Radding and Steinberg (1960).

Isolation of lipoproteins with $d < 1.006$ g/mL: 0.8 mL of lithium heparin plasma or serum was placed in a Quick–Seal® tube, overlaid with 1.2 mL of a $d = 1.006$ g/mL potassium bromide solution and submitted to ultracentrifugation for 2 hours and 12 minutes under the conditions described above. The upper layer was harvested by tube slicing at 11.5 mm and stored at −80°C until further analysis.

Isolation of lipoproteins with $d = 1.006$–1.019 g/mL: The remaining lower fraction of 1.2 mL was transferred to a new tube, adjusted to $d = 1.019$ g/mL by adding 48.6 µL of a $d = 1.34$ g/mL potassium bromide solution and made up to a final volume of 2 mL with a $d = 1.019$ g/mL potassium bromide solution. Centrifugation for 2 hours and 12 minutes was performed as described above and 0.8 mL of the supernatant was collected by tube slicing at 11.5 mm.

Isolation of lipoproteins with $d = 1.019$–1.063 g/mL: The remaining lower fraction of 1.2 mL was transferred to a new tube and mixed with 190.6 µL of a $d = 1.34$ g/mL potassium bromide solution and 609.4 µL of a $d = 1.063$ g/mL potassium bromide solution. The tube was heat–sealed and submitted to ultracentrifugation for 2 hours and 45 minutes under the same conditions as before. After centrifugation, 0.8 mL of the supernatant was collected by tube slicing at 11.5 mm.

Isolation of lipoproteins with $d = 1.063$–1.210 g/mL: The remaining lower fraction of 1.2 mL was transferred to a new tube and adjusted to $d = 1.210$ g/mL by adding 1'357 µL of a $d = 1.34$ g/mL potassium bromide solution. The sample was brought to a final volume of 2.6 mL with 43 µL of a $d = 1.210$ g/mL potassium bromide solution. Of this mixture, only 2 mL were submitted to ultracentrifugation for 2 hours and 12 minutes under similar conditions as before. 0.8 mL of the supernatant was collected by tube slicing, again at 11.5 mm.

Isolation of the lipoproteins with $d > 1.210$ g/mL: The remaining lower fraction of 1.2 mL gained from the step above was collected.

All fractions were stored at −80°C until further analysis.

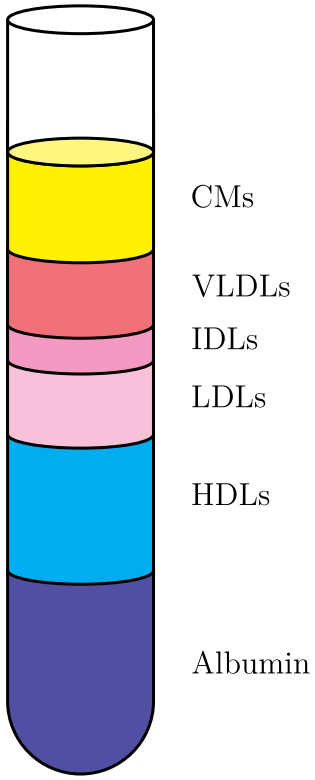


Figure 2.2: Hypothetical arrangement of lipoprotein fractions after ultracentrifugation.

2.1.2 Protocol B: Precipitation

A precipitation technique was applied to aliquots of the lithium heparin plasma and serum for separation of apoprotein B–containing lipoproteins, using a modified technique obtained by combining two protocols from Warnick et al. (1985) and Barrie et al. (1993a). As evaluated by previous studies, a heparin concentration of 1–5 mg/mL was used in the presence of 0.092M Mn^{2+} for optimal precipitation of apo B–containing lipoproteins.

Isolation of "Supernatant": 0.8 mL of plasma or serum was mixed with 80 μL of a heparin manganese chloride solution obtained by mixing 2.5 mL heparin–sodium injection solution (5,000 I.U./mL) with a 1.012 M manganese chloride solution, made up to 5 mL (resulting in a final Mn^{2+} concentration of 92 mM). After mixture, the sample was incubated at room temperature for 20 minutes and submitted to centrifugation at 4°C and 1500 g for 30 minutes with the supernatant being immediately removed after centrifugation.

Isolation of "Apo B–containing Precipitate": The remaining precipitate was dissolved in 0.25 mL of a 0.5 M sodium citrate and 1 mL of a 0.15 M sodium chloride solution.

All fractions were stored at −80°C until further analysis.

2.1.3 Estimation of Molecular Weights of Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis 10% (SDS–PAGE; Criterion TGX Stain–Free any kD, Bio–Rad, Switzerland) was used to identify protein–containing lipoprotein constituents of all fractions obtained as described in protocols A and B.

Before analysis, all fractions were desalted and concentrated by 10 kDa Amicon Ultra centrifugal filters (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) at 12,000 g and 4°C. The dialysis buffer consisted of 0.2 M sodium chloride and 0.3 mM EDTA.

After dialysis, all fractions showed pH values within normal limits (7–7.5) and protein concentration measurement was performed using NanoDrop (NanoDrop 1000, Thermo Scientific, Switzerland). If required, additional diluting steps were performed to adjust the protein amount to 5 μg protein per separated fraction before application to SDS–PAGE. Only lipoproteins with $d = 1.006$–1.019 g/mL obtained in protocol A had a lower protein amount with 2.5 μg protein per sample.

After adaption of protein amounts, all samples were diluted with "2x" Laemmli SDS–PAGE sample buffer (containing 62.5 mM Tris–HCl at pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% β–mercaptoethanol) in a proportion of 1:1 (v:v) and samples were heated at 95°C for 5 minutes. Appropriate volumes of samples were loaded on the gel together with a protein marker (Precision Plus Protein^{TM} All Blue Marker, Bio–Rad, Switzerland) and electrophoresis was performed using 200 V and 35 minutes run time. Afterwards, gels were activated and imaged through a ChemiDoc^{TM} System (Bio–Rad Clinical Diagnostics, Switzerland).

The relative migration distances (R_f) of the protein standards used and of the unknown proteins were calculated using the mobility of a protein (band), divided by the mobility of the ion front (R_f = distance to band / distance to dye front). Because the ion front was difficult to locate, mobilities were normalized to the tracking dye that migrates only

slightly behind the ion front, a technique proposed by Rodbard and Chrambach (1971).

The molecular weights of the unknown protein bands were then generated by the function $y = c \times \ln(x) + b$, using the logarithm of the molecular weights as a function of R_f (see graph plots in figures 3.2 and 3.4).

2.2 Lipidomics

The lipidomic study was performed between 2014–2015 at the Vetsuisse Faculty of the University of Zurich in collaboration with the Functional Genomics Center Zurich (FGCZ). Blood samples (lithium heparin plasma and serum) were taken from healthy Beagles before and after treatment with prednisolone. Lipid extraction was conducted using an MTBE (methyl *tert*–butyl ether) extraction protocol while analytical studies were performed by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) followed by data processing with Progenesis QI and statistical analyses by R.

Blood Samples

Six healthy Beagles, aged between 1 and 5 years (median 4) were included in this study. Gender was equally distributed with three dogs being male and three dogs being female (one spayed). All dogs were fed the same constant diet (Josera® Sensitive) at least one month before and during the study.

Before treatment with prednisolone, blood was collected into lithium heparin and serum tubes after at least 12 hours of fasting. Informed consent was obtained from the owners of the dogs. Their use was in accordance with the guidelines and directives established by the Animal Welfare Act of Switzerland (TVB NR 133/2013).

The dogs were treated on three consecutive days with prednisolone 50 mg once daily (Prednisolone 50 mg, Streuli Pharma AG, Uznach, Switzerland), administered orally. Immediately after treatment, blood from fasted dogs was collected again into lithium heparin and serum tubes. All samples were centrifuged at $1500 \times g$ for 10 minutes and stored at $-80°C$ until further analysis.

Reagents

Methanol (>99.9%), BHT (2,6–di–tert–butyl–4–methylphenol), MTBE (methyl *tert*–butyl ether, >99.8%), ammonium acetate (>98%) and LC–MS–grade water were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland).

Lipid Extraction

Lipid extraction was performed using a protocol developed by Matyash et al. (2008). Methanol (1.5 mL) was added to a 200 μL sample aliquot and the tube was vortexed. Then the sample was mixed with 5 mL of MTBE, including 500 μg/mL BHT as antioxidant, and the mixture was incubated for 1 hour at room temperature, gently shaking at 450 rpm.

Phase separation was induced by adding 1.25 mL of MS–grade water, sample was briefly vortexed and incubated 10 minutes at room temperature. After centrifugation at 1'000 *g* for 10 minutes, the upper (organic) phase (4.5 mL, MTBE/methanol/water 10:3:2.5, v/v/v) was collected, transferred into a new glass tube and dried under slow nitrogen flow on a thermoblock set at 37°C.

The dried sample was then dissolved in 500 μL of methanol (suitable for liquid chromatography) by shaking the glass tube at 300 rpm for 20 minutes, transferred into a specific glass vial and stored at −20°C until further analysis.

Liquid Chromatography – Mass Spectrometry

After storage, the dissolved lipid sample was vortexed briefly and 20 μL of the sample was diluted with 80 μL of a running buffer solution containing methanol (80%) and ammonium acetate (10 mM). From this mixture, 1 μL was injected into a LC–MS/MS system consisting of a Q–Exactive Hybrid Quadrupole–Orbitrap Mass Spectrometer (Thermo Scientific) combined with an in house packed tip column (C18 HSST Waters). Samples were run in randomized order.

Data Processing and Lipid Identification

Data processing was performed by importing raw files from LC–MS/MS into Progenesis–QI (Nonlinear Dynamics, Newcastle upon Tyne, U.K.), used for automatic alignment, denoising, deconvolution and extraction of ion peaks. Fragments (such as H^+, Na^+, $NH_4{}^+$, $HCOO^-$, CH_3COO^-) commonly added to lipid molecules during analysis were taken into consideration.

Every feature found during mass spectrometry was attached to the best fitting lipid molecular species available on the Lipid Maps database with lipid ions being first identified by their mass to charge ratio (m/z) by Lipid Maps (Sud et al., 2007), currently the most detailed lipid database available. Second, due to multiple identifications of specific lipid molecular species, further lipid identification was performed on an in–house built Pyton script (Dr. Jeremy Deuel, University Hospital, Zurich) to enhance the accuracy of lipid identification by setting the fragmentation score to >0, the overall score of every feature was set to >2 × standard deviation (SD) and isotope similarity to >2 × SD.

Taking these precautions, lipids were considered to be securely identified and the common standard lipid language described by Lipid Maps was applied.

Statistical Analysis

Data was normalized by logarithmic transformation of raw data (relative abundance of lipid ions), indicating normal distribution of lipids within dogs (QQ–plots).

Lipids from dogs before prednisolone treatment were analyzed by paired Student's *t*–test to compare lithium heparin plasma with serum.

All identified lipids were further analyzed using the paired Student's t-test for comparison between before and after prednisolone treatment, separately performed for lithium heparin plasma and serum.

The statistical analyses and graphics were performed using the open source program R (R Core Team, 2015) and all p values were corrected for multiple testing (false discovery rate). Differences were considered significant at values of $p \leq 0.05$.

Chapter 3

Results

3.1 Lipoprotein Separation

3.1.1 Protocol A: Ultracentrifugation

SDS–PAGE analysis of the five fractions (d <1.006 g/mL, d 1.006–1.019 g/mL, d 1.019–1.063 g/mL, d 1.063–1.210 g/mL, d >1.210 g/mL) from lithium heparin plasma revealed five bands in fraction 1, two bands in fraction 2, two bands in fraction 3, one band in fraction 4 and five bands in fraction 5 as the most intense protein stains (figure 3.1, Plasma).

SDS–PAGE analysis of the five fractions (d <1.006 g/mL, d 1.006–1.019 g/mL, d 1.019–1.063 g/mL, d 1.063–1.210 g/mL, d >1.210 g/mL) from serum revealed four bands in fraction 1, two bands in fraction 2, two bands in fraction 3, one band in fraction 4 and five bands in fraction 5 as the most intense protein stains (figure 3.1, Serum).

The estimated molecular weights of protein bands found on SDS–PAGEs produced by protocol A from lithium heparin plasma and serum are shown in Fig. 3.2. A plot of migration versus logarithmic molecular weight shows logarithmic function for all proteins found.

Further, all estimated molecular weights found in lithium heparin plasma and serum are summarized in table 3.1. Possible relations with apoproteins will be discussed in the next chapter.

Table 3.1: Estimated molecular weights of isolated lipoprotein fractions from lithium heparin plasma and serum using protocol A as described in subsection 2.1.1.

Lane	Density Fraction	Molecular Weights (kDa) in Plasma	Molecular Weights (kDa) in Serum
1	<1.006 g/mL	67, 52, 33, 30, 23	58, 34, 31, 23
2	1.006–1.019 g/mL	250, 23	298, 23
3	1.019–1.063 g/mL	23, 14	23, 15
4	1.063–1.210 g/mL	23	23
5	>1.210 g/mL	75, 60, 44, 31, 26	75, 56, 51, 31, 26

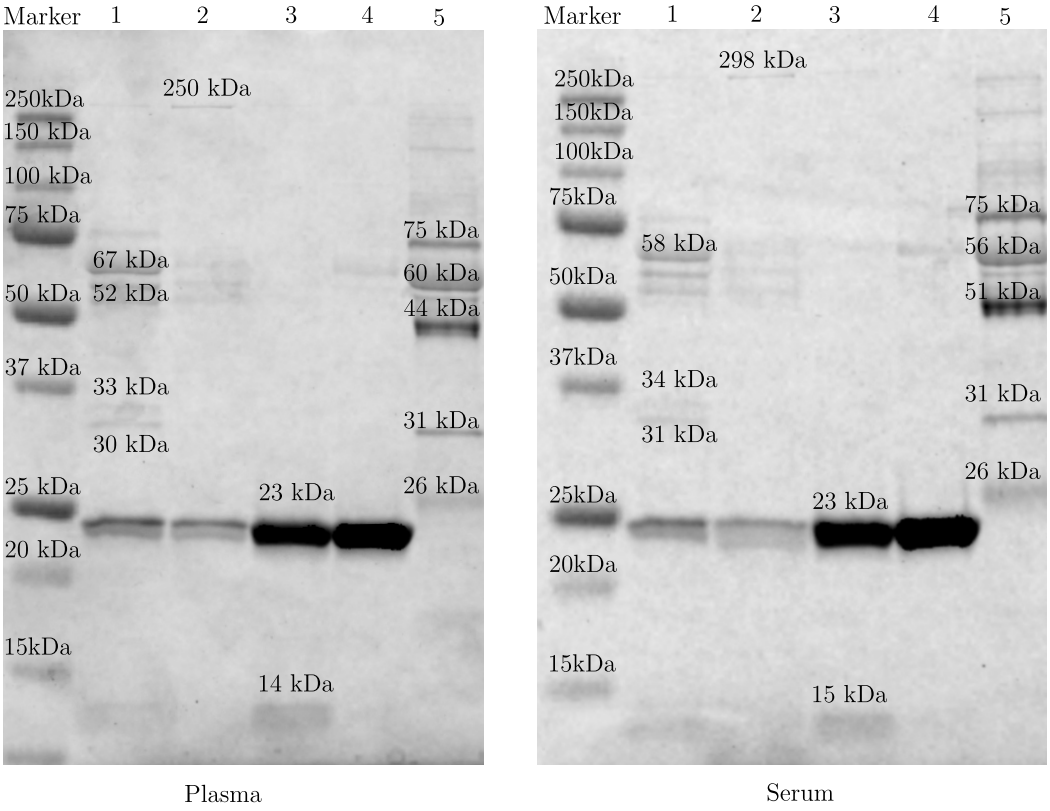
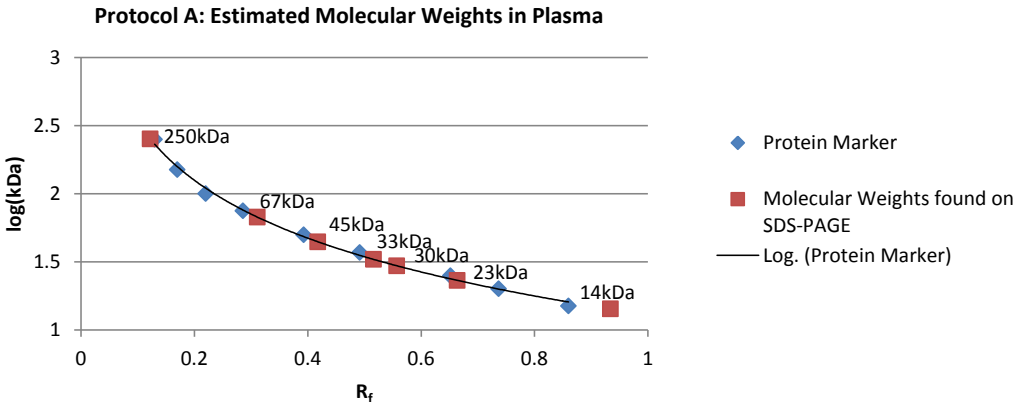
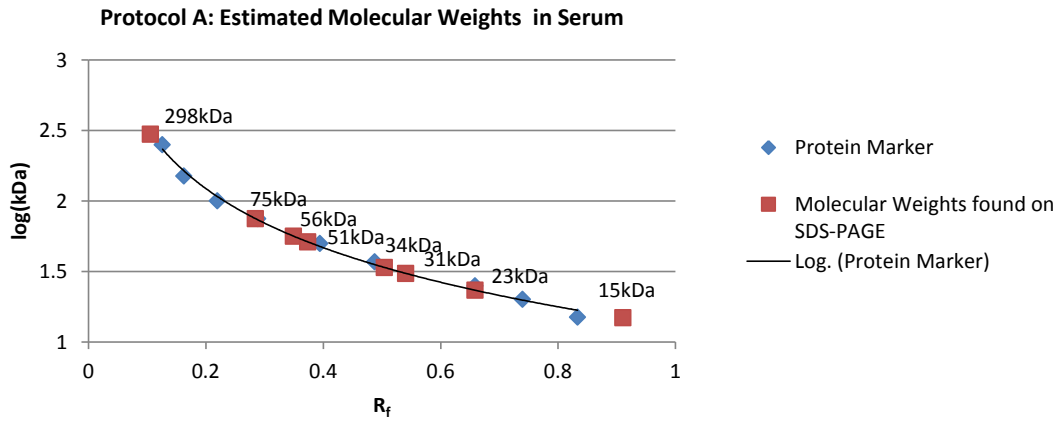


Figure 3.1: SDS–PAGEs of canine lithium heparin plasma and serum, lipoproteins were separated by protocol A. Description of lanes: 1) d <1.006 g/mL; 2) d 1.006–1.019 g/mL; 3) d 1.019–1.063 g/mL; 4) d 1.063–1.210 g/mL; 5) d >1.210 g/mL. Protein marker with defined standard molecular weights ranging from 10 to 250 kDa was applied (Marker), each lane consists of 5 μg total protein, except lane 2 (2.5 μg).



(a) Plasma



(b) Serum

Figure 3.2: Molecular weight estimations of major protein bands observed on SDS–PAGEs after lipoprotein separation according to protocol A.

3.1.2 Protocol B: Precipitation

Serum samples precipitated more quickly during analytical procedures and in greater amounts than lithium heparin plasma. However, no obvious turbidity was seen in the supernatant of either blood sample as an indication for incomplete precipitation.

SDS–PAGE analysis of the two fractions from lithium heparin plasma revealed six bands in the precipitate and six bands in the supernatant as the most intense protein stains (figure 3.3, Plasma).

SDS–PAGE analysis of the two fractions from serum revealed six bands in the precipitate and six bands in the supernatant as the most intense protein stains(figure 3.3, Serum).

The estimated molecular weights of protein bands detected on both SDS–PAGEs are shown in Fig. 3.4. A plot of migration versus logarithmic molecular weight shows a logarithmic function for all proteins.

All estimated molecular weights found on SDS–PAGEs from lithium heparin plasma and serum are summarized in table 3.2. Possible relations with apoproteins will be discussed in the next chapter.

Table 3.2: Estimated molecular weights of isolated precipitate and supernatant from lithium heparin plasma and serum using protocol B.

Fraction	Molecular Weights (kDa) in Plasma	Molecular Weights (kDa) in Serum
Precipitate	255, 63, 56, 49, 33, 23	266, 75, 61, 49, 35, 25
Supernatant	75, 63, 49, 33, 26, 23	75, 61, 49, 35, 26, 25

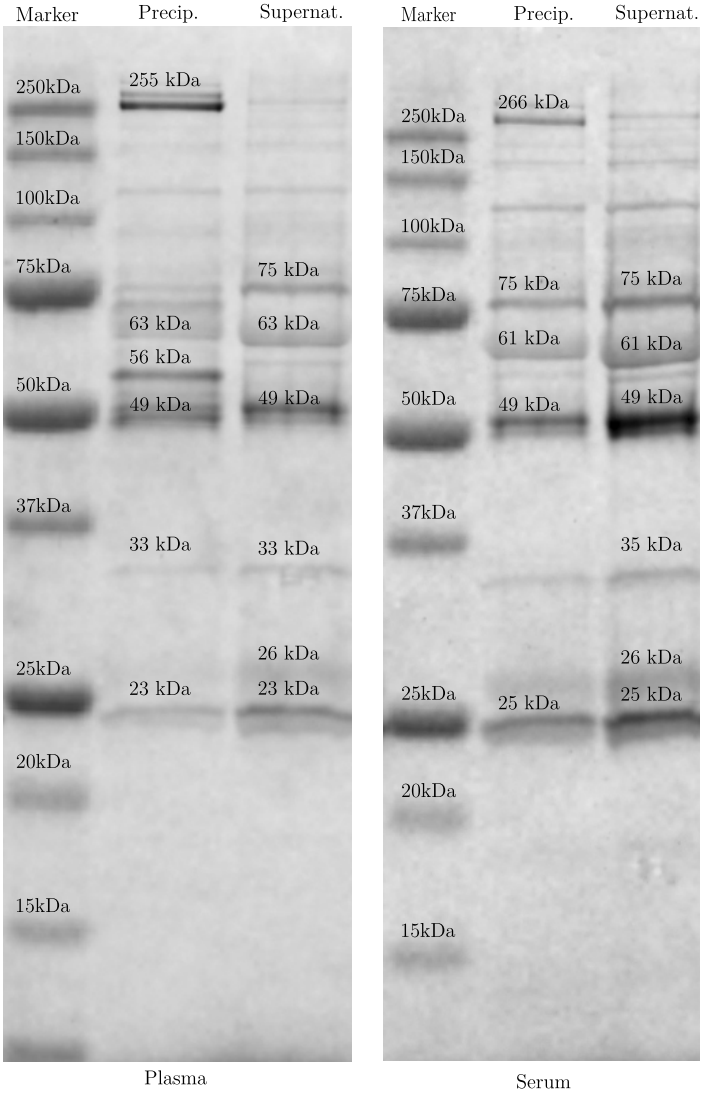
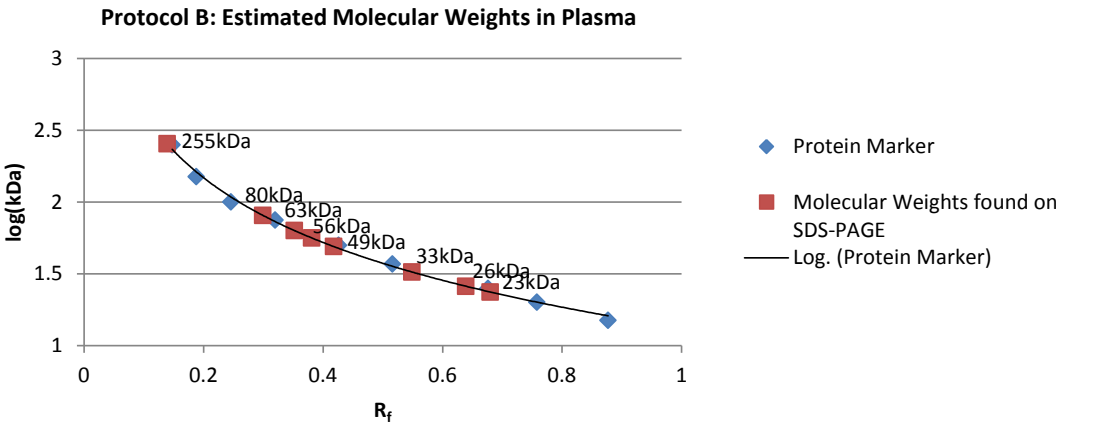
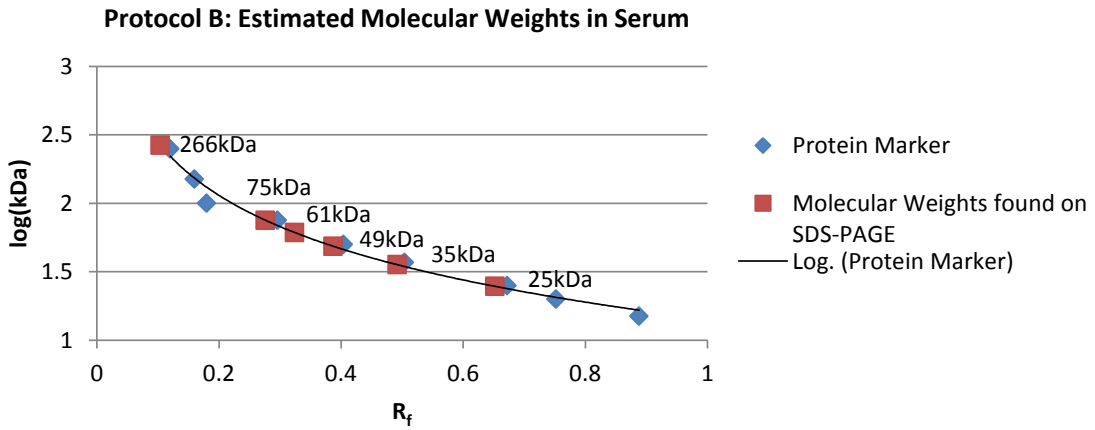


Figure 3.3: SDS–PAGEs of canine lithium heparin plasma and serum, lipoproteins were separated by protocol B. Description of lanes: Precip. = Precipitate; Supernat. = Supernatant. Protein marker with defined standard molecular weights ranging from 10 to 250 kDa was applied (Marker), each lane consists of 5 μg total protein.



(a) Plasma



(b) Serum

Figure 3.4: Molecular weight estimations of major protein bands observed on SDS–PAGEs after lipoprotein separation according to protocol B.

3.2 Lipidomics

3.2.1 Lipid Identification and Distribution

Canine Plasma

408 different lipids were identified in the canine lithium heparin plasma and categorized into seven categories as shown in table 3.3.

The highest amount of identified lipid molecular species found was the fatty acyls (FAs), at 44.6%, followed by glycerophospholipids (GPs, 19.4%), sterol lipids (STs, 10.5%), glycerolipids (GLs, 8.8%), sphingolipids (SPs, 7.1%), prenol lipids (PRs, 6.9%) and polyketides (PKs, 2.7%). Of the eight main lipid categories as categorized by Lipid Maps, only saccharolipids (SLs) were missing.

Canine Serum

In all, 399 different lipids were identified in the canine serum and categorized into eight categories of lipids, as shown in table 3.3.

The highest amount of identified lipid molecular species found was the fatty acyls (FAs), at 47.4%, followed by the glycerophospholipids (GPs, 19.3%), sterol lipids (STs, 10.3%), sphingolipids (SPs, 7.8%), glycerolipids (GLs, 7.0%), prenol lipids (PRs, 5.8%), polyketides (PKs, 2.3%) and one saccharolipid (SL, 0.3%).

A complete data set of the canine lipidome (561 molecular lipid species) found in plasma and serum during this study is presented in table A.1 in the appendix, with further details on all identified lipids available on the Lipid Maps homepage (www.lipidmaps.org).

Table 3.3: Lipid categories and classes analyzed in canine plasma and serum.

Lipid Category	Main Class	Lipid Species Plasma	Lipid Species Serum
Fatty Acyls (FAs)		**182**	**189**
	Other Fatty Acyls	1	0
	Fatty Acids and Conjugates	94	80
	Octadecanoids	19	16
	Eicosanoids	8	15
	Docosanoids	3	3
	Fatty Alcohols	7	9
	Fatty Aldehydes	5	8
	Fatty Esters	25	34
	Fatty Amides	12	12
	Hydrocarbons	2	3
	Oxygenated Hydrocarbons	6	8
Glycerophospholipids (GPs)		**79**	**77**
	Other Glycerophospholipids	1	0
	Glycerophosphocholines (PCs)	29	26
	Glycerophosphoethanolamines (PEs)	17	18
	Glycerophosphoserines (PSs)	10	14
	Glycerophosphoglycerols (PGs)	1	1
	Glycerophosphoinositols (PIs)	8	8
	Glycerophosphates (PAs)	10	8
	Oxidized Glycerophospholipids	3	2
Sterol Lipids (STs)		**43**	**41**
	Sterols	18	17
	Steroids	3	5
	Secosteroids	8	10
	Bile Acids and Derivates	12	7
	Steroid Conjugates	2	2
Glycerolipids (GLs)		**36**	**28**
	Monoradylglycerols	6	5
	Diradylglycerols	22	20
	Triradylglycerols	8	3
Sphingolipids (SPs)		**29**	**31**
	Sphingoid Bases	5	9
	Ceramides	9	11
	Phosphosphingolipids	8	6
	Neutral Glycosphingolipids	4	2
	Acidic Glycosphingolipids	3	3
Prenol Lipids (PRs)		**28**	**23**
	Isoprenoids	26	20
	Quinones and Hydroquinones	2	2
	Hopanoids	0	1
Polyketides (PKs)		**11**	**9**
	Linear Polyketides	0	1
	Macrolides and Lactone Polyketides	2	2
	Flavonoids	8	6
	Aromatic Polyketides	1	0
Saccharolipids (SLs)		**0**	**1**
	Other Acyl Sugars	0	1

3.2.2 Plasma vs. Serum

246 molecular lipid species were identified in both plasma and serum in all six dogs.

When these individual lipids are compared across the matrices, out of the 246, eleven lipids (4.5%) were significantly altered in their relative mean abundances between plasma and serum as analyzed by the paired Student's *t*–test, graphically illustrated by a volcano plot (figure 3.5) and summarized in table 3.4.

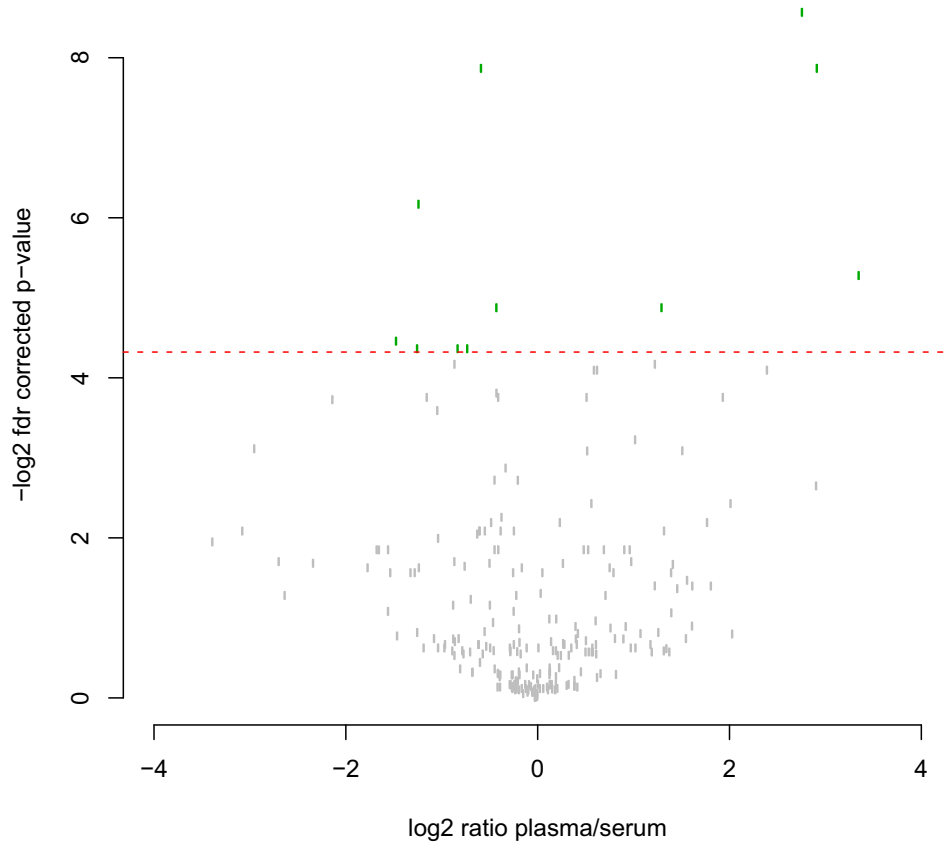


Figure 3.5: Volcano plot showing eleven statistically significant different lipids (green strokes) between canine lithium heparin plasma and serum.

Q–Q–plots for plasma and serum are shown in section A.2 in the appendix. To summarize, seven fatty acyls (FAs) were found to be significantly altered, followed by two prenol lipids (PRs), one glycerophospholipid (GP) and one polyketide (PK). Ratios were calculated for all significantly changed lipids, comparing plasma with serum to express the magnitude of variation. Four lipids were increased in plasma, with ratios between 1.3 to 3.4, and seven lipids were increased in serum, with ratios between – 0.6 to – 1.2 (plasma / serum).

Table 3.4: Eleven significantly different lipids comparing lithium heparin plasma to serum by paired Student's *t*–test.

Nr.	Cat.	Main Class	Sub Class	Common Name	Systematic Name	Log2 Ratio Plasma /Serum	*p*
1	FA	Fatty Acids and Conjugates	Dicarboxylic acids	–	2E,4E,8E,10E–Dodecatetraenedioic acid	– 1.2	0.014
2	FA	" "	Hydroxy fatty acids	3–hydroxy–isoheptanoic acid	3–hydroxy–6–methyl–hexanoic acid	– 1.2	0.049
3	FA	" "	" "	–	11S–hydroxy–tetradecanoic acid	– 0.8	0.049
4	FA	" "	Oxo fatty acids	14–keto pentadecanoic acid	14–oxo–pentadecanoicacid	2.8	0.003
5	FA	" " or Docosanoids	Unsaturated fatty acids or –	Adrenic Acid	7Z,10Z,13Z,16Z–docosatetraenoic acid	– 0.6	0.004
6	FA	Fatty amides	N–acyl amines	N–palmitoyl glycine	N–hexadecanoyl–glycine	– 1.4	0.046
7	FA	Fatty esters	Wax monoesters	–	formyl 7–oxo–11E–tetra–decenoate	– 0.4	0.034
8	GP	Glycerophos–phoserines	1–(1Z–alkenyl),2–acylglycero–phosphoserines	PS(P–16:0/18:0)	1–(1Z–hexadecenyl)–2–octadecanoyl–glycero–3–phosphoserine	1.3	0.034
9	PK	Flavonoids	Chalcones and dihydrochalcones	–	1–(2,5–dihydro–6,8–dihydroxy–3–methyl–1–benzoxepin–7–yl)–3–phenyl–1–propanone	3.4	0.026
10	PR	Isoprenoids	C15 isoprenoids (sesquiterpenes)	(+)–Blennin D	–	2.9	0.004
11	PR	Isoprenoids	Retinoids	9–cis–retinol	9Z–retinol	– 0.7	0.049

3.2.3 Prednisolone Treatment

Canine Plasma

Ten different lipids turned out to be significantly decreased or increased in canine plasma after treatment with prednisolone (figure 3.6 and table 3.5).

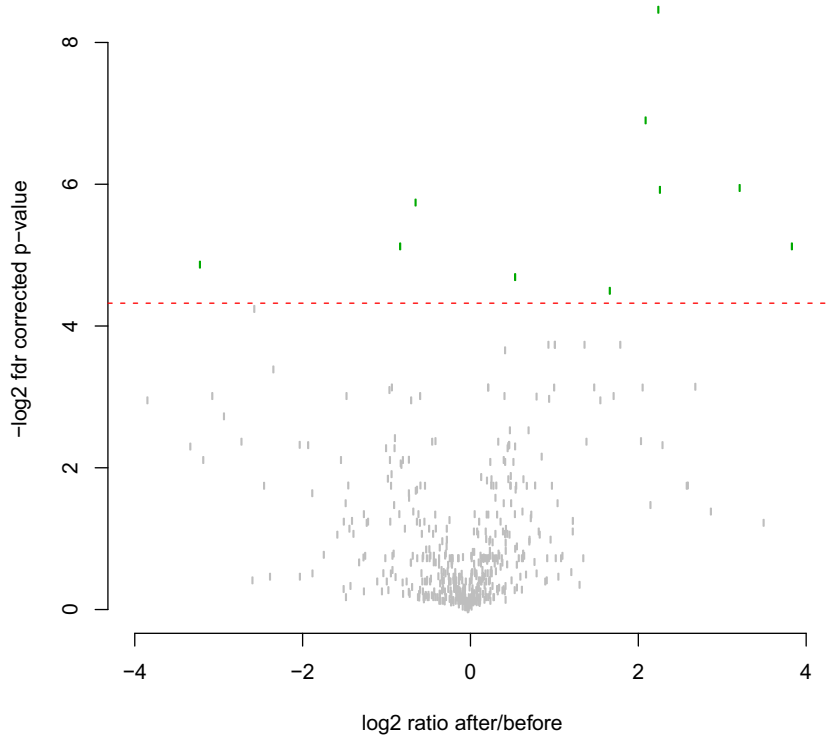


Figure 3.6: Volcano plot showing statistically significant lipids (green strokes) in plasma after prednisolone treatment.

Ratios were calculated for all significantly changed lipids after prednisolone treatment to express the magnitude of variation. In summary, three lipids were decreased after prednisolone treatment, with ratios between – 0.6 to – 3.2, and seven lipids were enhanced after treatment, with ratios between 0.6 to 3.9.

All significantly altered lipids after prednisolone treatment are illustrated in figure 3.7, showing their abundances within each dog according to color intensity, with blue indicating a decreased amount and red indicating an increased amount of the lipid in question. Q–Q–plots for plasma are shown in section A.3 in the appendix at the end of this manuscript.

Table 3.5: Ten statistically significant lipids in plasma after prednisolone treatment.

Nr.	Cat.	Main Class	Sub Class	Common Name	Systematic Name	Log2 Ratio After/ Before	p
1	FA	Fatty Acids and Conjugates	Branched fatty acids	Hydroxyphthioceranic acid (C39)	2S,4S,6S,8S,10R,12R, 14R–heptamethyl–15–hydroxy–dotriacontanoic acid	– 3.2	0.034
2	FA	” ”	Hydroxy fatty acids	–	3–hydroxy–hexadecanoic acid	2.3	0.017
3	FA	” ”	Unsaturated fatty acids	–	10,13,16–Docosatriynoic acid	3.9	0.029
4	FA	” ”	” ”	–	8,11–Eicosadiynoic acid	1.7	0.044
5	FA	” ”	” ”	–	11Z–octadecen–9–ynoic acid	– 0.8	0.029
6	FA	Docosanoids	–	DPA	7Z,10Z,13Z,16Z,19Z–docosapentaenoic acid	3.2	0.016
7	FA	Octadecanoids	Other Octadecanoids	–	9R–hydroxy–octadecanoic acid	2.3	0.003
8	FA	” ”	Other Octadecanoids	–	3R–hydroxy–octadecanoic acid	2.1	0.008
9	GP	Glycerophosphoethanolamines	1–(1Z–alkenyl),2–acyl–glycerophosphoethanolamines	PE(P–16:0/22:4)	1–(1Z–hexadecenyl)–2–(7Z,10Z,13Z,16Z–docosatetraenoyl)–glycero–3–phosphoethanolamine	0.6	0.039
10	GL	Monoradylglycerols	Monoacylglycerols	MG(18:1(11E)/0:0/0:0)[rac]	1–(11E–octadecenoyl)–rac–glycerol	– 0.6	0.019

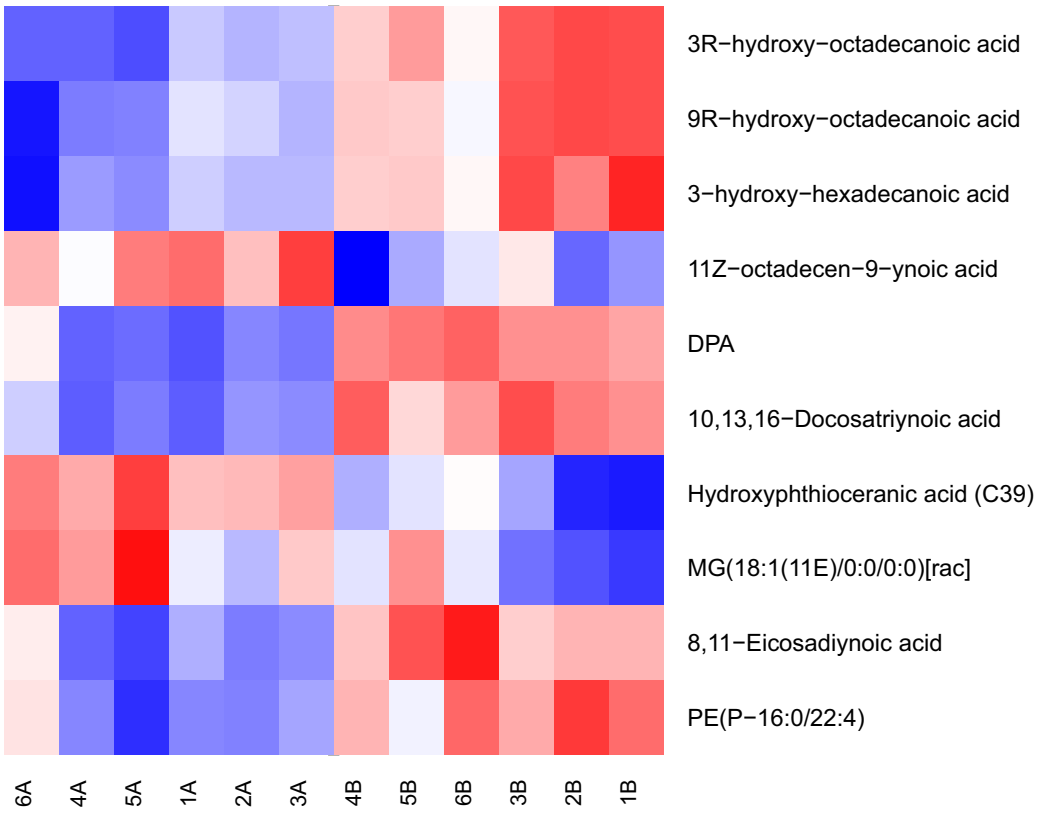


Figure 3.7: Heatmap of statistically significant lipids in canine lithium heparin plasma compared before (A) and after (B) prednisolone treatment. Blue indicates a decreased amount and red and increased amount of this lipid.

Canine Serum

Three different lipids turned out to be significantly decreased or increased in canine serum after treatment with prednisolone (figure 3.8 and table 3.6).

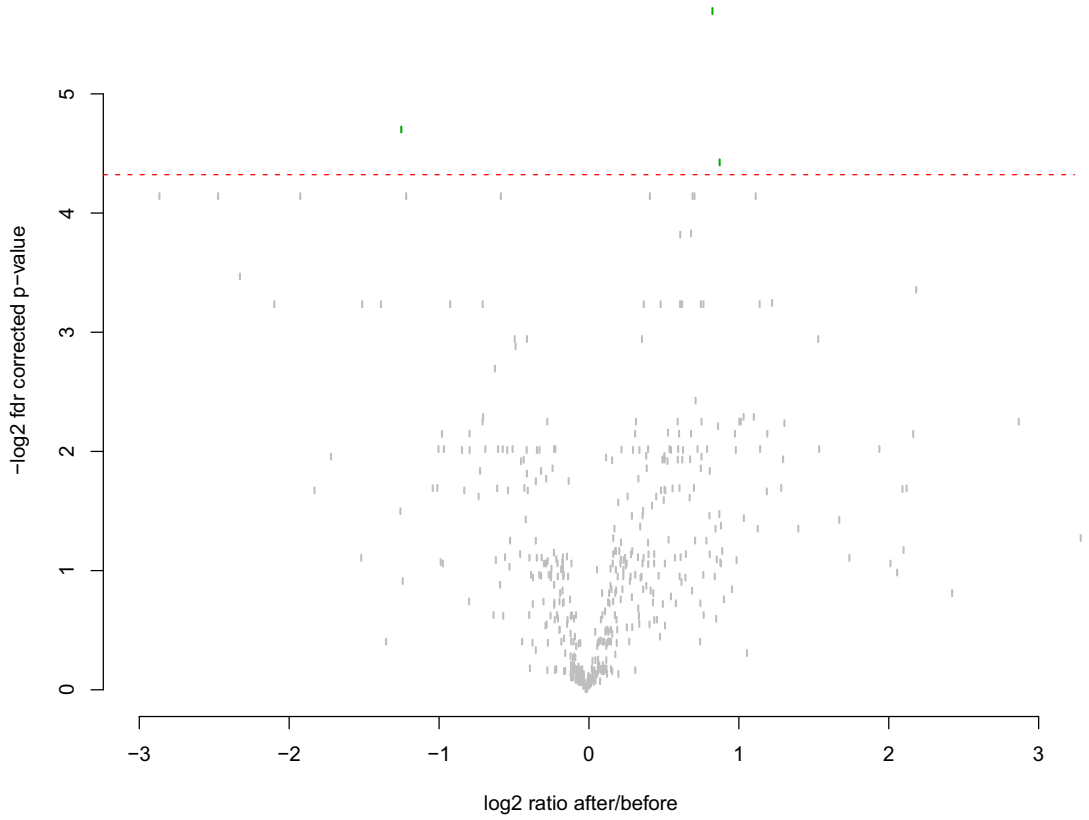


Figure 3.8: Volcano plot showing statistically significant lipids (green strokes) in serum after prednisolone treatment.

Ratios were calculated for all significantly changed lipids after prednisolone treatment to express the magnitude of variation. To summarize, one lipid was decreased after prednisolone treatment, by a ratio of –1.2, and two lipids were increased after treatment, by ratios of 0.8 to 0.9. Q–Q–plots for serum are shown in section A.3 in the appendix at the end of this manuscript.

In addition, all significantly altered lipids after prednisolone treatment are illustrated in figure 3.9, which their abundances within each dog by means of color intensity, with blue indicating a decreased amount and red indicating an increased amount of a lipid.

Interestingly, 9R–hydroxy–octadecanoic acid was found to be significantly altered after prednisolone treatment in both serum and plasma. It was 0.9 times higher in serum and 2.3 times higher in plasma.

Table 3.6: Statistically significant lipids in serum after prednisolone treatment.

Nr.	Cat.	Main Class	Sub Class	Common Name	Systematic Name	Log2 Ratio After/ Before	p
1	FA	Octadeca–noids	Other Octade–canoids	–	9R–hydroxy–octadecanoic acid	0.9	0.047
2	FA	Fatty Acids and Con–jugates or Docosanoids	Unsaturated fatty acids or –	Adrenic acid	7,10,13,16–docosatetraenoic acid	0.8	0.019
3	GP	Glycerophos–phoethanol–amines	Diacylglycero–phosphoethanol–amines	PE(20:4/20:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–2–eicosanoyl–glycero–3–phosphoethanolamine	–1.2	0.038

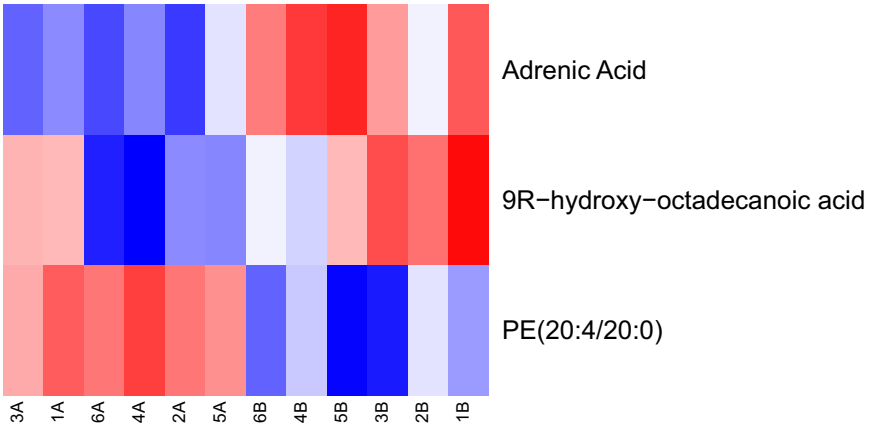


Figure 3.9: Heatmap of statistically significant lipids in canine serum before (A) and after (B) prednisolone treatment.

Chapter 4

Discussion

4.1 Lipoprotein Separation

4.1.1 Protocol A: Ultracentrifugation

Lipoprotein separation with Protocol A was performed according to human lipoprotein density classes, with density (d) fraction <1.006 g/mL representing human VLDLs, d = 1.006–1.019 g/mL human IDLs, d = 1.019–1.063 g/mL human LDLs, d = 1.063–1.210 g/mL human HDLs and d >1.210 g/mL no specific fraction.

Separation of lipoprotein fractions was evaluated according to the estimated molecular weights from SDS–PAGEs, which were assumed to represent related apoproteins. For protocol A, the most suitable apoproteins in the dogs are shown in table 4.1 for plasma and in table 4.2 for serum. However, as SDS–PAGEs show not only apoproteins but all proteins within a fraction, the estimated molecular weights could also be indicative of various other proteins.

Table 4.1: Assumed apoproteins isolated from plasma using protocol A.

Density Fraction	Molecular Weights (kDa) in Plasma	Indicated Apoproteins
<1.006 g/mL	67, 52, 33, 30, 23	Albumin, ?, E, D, A–I
1.006–1.019 g/mL	250, 23	B–48, A–I
1.019–1.063 g/mL	23, 14	A–I, A–II
1.063–1.210 g/mL	23	A–I
>1.210 g/mL	75, 60, 44, 31, 26	?, Albumin, A–IV, D/E, ?

An intense protein band with an estimated molecular weight of ≈ 23 kDa, indicative of apo A–I, was found in both canine plasma and serum. This is in agreement to another study with canine apoproteins, where a protein band of 25 kDa was identified as apo A–I by LC–MS (Asuka et al., 2013). This protein band was most intense and broad in the two

Table 4.2: Assumed apoproteins isolated from serum using protocol A.

Density Fraction	Molecular Weights (kDa) in Serum	Indicated Apoproteins
<1.006 g/mL	58, 34, 31, 23	Albumin, E, D, A–I
1.006–1.019 g/mL	298, 23	B–48, A–I
1.019–1.063 g/mL	23, 15	A–I, A–II
1.063–1.210 g/mL	23	A–I
>1.210 g/mL	75, 56, 51, 31, 26	?, Albumin, A–IV, D/E, ?

fractions covering *d* 1.019–1.210 g/mL, which most closely represent the density range of canine HDL (Xenoulis and Steiner, 2010; Bauer, 2004), verifying apo A–I as the major apoprotein of HDL.

However, the suspected apo A–I was also found in fractions with lower densities from *d* <1.006 g/mL up to 1.019 g/mL, where no canine HDL was expected. Therefore either the separation of lipoprotein fractions was inaccurate, possibly due to an insufficient run time, or another protein has similar molecular weights to apo A–I. Apo A–I could also occur separated from HDL due to ultracentrifugal forces (Munroe et al., 2015), however labile apoproteins, such as Apo A–I and apo E, have been reported to be found at the bottom of the tube after centrifugation, hence in a fraction above HDL, such as *d* >1.210g/mL (Brousseau et al., 1993; Kunitake and Kane, 1982).

Overlapping densities of canine LDL, *d* 1.019–1.087 g/mL, and HDL, *d* 1.025–1.210 g/mL (Mahley and Weisgraber, 1974), can best be seen in the fraction of *d* 1.019–1.063 g/mL, with strong protein bands indicative of apo A–I and faint bands assumed to represent apo B–48 with molecular weights >250 kDa. The faint bands may be due to technical aspects, as only 5μg protein per lane was applied on our SDS–PAGEs, and apo A–I occurs at a higher percentage than apo B–48 and possibly apo B–100.

Apo B–48 was also assumed to occur in the fraction of *d* 1.006–1.019 g/mL, which represents human IDLs (Jonas, 2002) and has only occasionally been reported for dogs (Bauer, 2004). However, lipoproteins have been found in this fraction and the occurrence of apo B–48 was previously reported for canine CMs, VLDLs and LDLs, (Xenoulis and Steiner, 2010). Therefore, either canine IDL does exist or VLDLs and LDLs are not separated as accurately as previously reported (Mahley and Weisgraber, 1974), which seems plausible regarding the ongoing exchange of molecules in lipoprotein metabolism. However, in contrast to human studies, we report a protein band indicative of apo B–48 instead of apo B–100 within the IDL fraction.

Protein bands of 30–31 kDa and 33–34 kDa, indicative of apo D and E, were found in the first fraction (*d* <1.006 g/mL)L. However, mainly apo E has been mentioned so far in dogs, and then especially in a cholesteryl ester–rich subfraction of HDL that shows a similar density to LDL (Barrie et al., 1993a). However, we did not find protein bands indicative of apo E in fractions representing canine HDL. But looking at the last fraction with *d* >1.210 g/mL, the protein band of $\approx$ 31 kDa was found, indicative of either apo

D or E. This could either represent a separated apoprotein from HDL or no apoprotein at all. Instead, another protein could represent this band, e.g. haptoglobin, identified at a molecular weight of 35 kDa in another study with canine lipoproteins (Asuka et al., 2013).

Further, an established apoprotein of HDL, apo A–IV, may correspond to protein bands of 44–51 kDa. Apo A–IV was detected in humans at a weight of 44–46 kDa (Jonas, 2002). However, in our study, they were primarily detected at d >1.210 g/mL, when the expected density for canine HDL lies lower at d 1.025–1.210 g/mL (Mahley and Weisgraber, 1974). This may be due to a false identification of this protein band or the disconnection from HDL and therefore occurring in a higher density class because of ultracentrifugal forces, although apo E and A–I would then be expected to be found in the same fraction. It is also possible that apo A–IV is associated with heavier HDLs as reported for various species including the dog in another study (Hollanders et al., 1986). However, that study hypothesized that apo E and apo A–IV do not coexist on the same HDL, which would be different to our findings. Additionally, the protein bands of 44–51 kDa were also detected as faint bands at the fraction with d <1.006 g/mL, indicative of CMs. As apo A–IV was already reported for CMs in humans (Jonas, 2002), this could be true for dogs as well. However, CMs were thought to be limited due to fasting.

Apo A–II was interpreted into faint protein bands of 14-15 kDa, occurring in two fractions in our study: d <1.006 g/mL and d 1.019-1.063 g/mL. The latter seems reasonable, as apo A–II is related to HDL in humans, making it plausible to have the same characteristics in dogs. But apo A–II appearing in the fraction related to canine VLDL was not reported before, which may be due to the newer imaging techniques for SDS–PAGEs, which are more sensitive than the traditional staining methods. However, false identification could always have occurred and this molecular weight could also represent another protein, e.g. serum amyloid A, an acute phase protein found at 13 kDa within canine plasma (Chikamune et al., 1998). Although apo A–II is considered to be one of the major apoproteins of HDL in humans, it was only reported once before for canine lipoproteins by Puppione et al. (2008). That study confirmed its presence and indicated a possible connection with canine HDL_1. This would match our results with a protein band of 14–15 kDa in the fraction of d 1.019–1.063 g/mL.

Further, lithium heparin plasma as well as serum showed several proteins strongly visible in the last fraction of d >1.210 g/mL, assumed to represent contamination with other proteins as previously reported (Chikamune et al., 1998). One of these proteins has a strong band of ≈ 75 kDa, occurring in the density fraction >1.210 g/mL, not currently related to any known apoprotein in dogs. Hypothetically, it may represent clusterin (70 kDa), a complement lysis inhibitor and also known as apoprotein J, reported as forming complexes with apo A–I in human HDLs (Jenne et al., 1991). However, apo A–I does not seem to be present in this fraction. Another explanation for a protein of ≈ 75 kDa would be the occurrence of hemoglobin (≈ 65 kDa), because an orange band was visible during the analytical procedure at the very bottom of the ultracentrifugation tube, previously reported from the hemoglobin of rats (Rodriguez-Sureda et al., 2002). Transferrin has also been reported to have a molecular weight of ≈ 80 kDa and was shown to be enriched in small and dense subfractions of HDL (HDL_3), which means it could also represent the

Table 4.3: Isolated fractions, expected lipoproteins and indicated apoproteins on SDS–PAGEs.

Isolated Fraction	Human Lipo–proteins	Expected Canine Lipoproteins	Expected Canine Apoproteins	Aporoteins indicated on SDS–PAGEs
<1.006 g/mL	VLDL	VLDL	B–100, B–48, E, C	(B–48), A–I, (A–II), E, D
1.006–1.019 g/mL	IDL	-	–	B–48, A–I
1.019–1.063 g/mL	LDL	LDL, HDL_1	B–48, A, E, C	(B–48), A–I, A–II
1.063–1.210 g/mL	HDL	Rest of LDL and HDL_{1-3}	B–100, B–48, A, E, C	A–I
>1.210 g/mL	>HDL	-	–	A–IV, D/E

protein band at ≈ 75 kDa (McPherson et al., 2007).

No apo C (7–9 kDa) was indicated in our study, probably due to the dialysis of samples with 10 kDa filters, and thus losing proteins with molecular weights <10 kDa.

In conclusion, canine lipoprotein separation probably needs longer ultracentrifugal run times than used in this study, but such an increase may further contribute to the lability of apoproteins and allow more time for oxidation. Further, slightly different molecular weights than those reported previously for canine apoproteins as well as small differences between plasma and serum occurred with this protocol, possibly due to analytical procedures and manual estimation of molecular weights. Nevertheless, our results in this study were similar to those reported previously for canine lipoproteins (Mahley and Weisgraber, 1974; Bauer, 2004), verifying different density ranges for canine lipoproteins compared with human lipoproteins and indicating overlapping lipoprotein densities. Most apoproteins assumed to be found in this protocol were comparable to those found in another study investigating canine lipoproteins and analyzing apoproteins either by SDS–PAGE (Chikamune et al., 1998) or mass spectrometry (Asuka et al., 2013).

4.1.2 Protocol B: Precipitation

Similar to the overlapping lipoprotein densities in dogs, lipoprotein (a) in humans, an LDL–like lipoprotein containing apo B and apo (a), has a density range overlapping with HDL (Warnick and Albers, 1978), requiring different separation techniques, such as precipitation.

The protocol we used here consisted of 92 mM heparin manganese chloride, which was reported to precipitate apoprotein B–containing lipoproteins (Warnick et al., 1985) in a human study, and similarly to another study with canine lipoproteins (Barrie et al., 1993a). The heparin manganese chloride technique is also the method recommended by the International Federation of Clinical Chemistry, although an ultracentrifugation step is generally a precondition for excluding VLDLs for cholesterol measurements of LDLs

and HDLs. During our study, we evaluated precipitation with and without a previous ultracentrifugation step for removal of VLDLs (d <1.006 g/mL), resulting in only minor differences. Because the same protocol for removal of VLDLs was used as described above (4.1.1) in protocol A, removal of VLDLs seemed to either be incomplete or contaminated with other proteins. For this reason, we only present precipitation without previous ultracentrifugation in this study.

Table 4.4: Estimated molecular weights and assumed related proteins using protocol B in plasma.

Fraction	Molecular Weights (kDa) in Plasma	Indicated Apoproteins
Precipitate	255, 63, 56, 49, 33, 23	B–48, (Albumin), ?, A–IV, E/D, A–I
Supernatant	75, 63, 49, 33, 26, 23	?, (Albumin), A–IV, E/D, ?, A–I

Table 4.5: Estimated molecular weights and assumed related proteins using protocol B in serum.

Fraction	Molecular Weights (kDa) in Serum	Indicated Apoproteins
Precipitate	266, 75, 61, 49, 35, 25	B–48, ?, (Albumin), A–IV, E/D, A–I
Supernatant	75, 61, 49, 35, 26, 25	?, (Albumin), A–IV, E/D, ?, A–I

The precipitated fractions showed strong protein bands around ≈ 260 kDa, indicative of apo B–48 in both lithium heparin plasma and serum. However, the same protein band was very faintly visible in the remaining supernatant as well. This is in contrast to two previous studies with canine lipoproteins (Barrie et al., 1993a; Asuka et al., 2013), which both reported almost complete precipitation, although the latter used a higher amount of manganese chloride.

In our study, incomplete precipitation could have occurred due to absent removal of triglyceride–rich VLDLs as hypothesized by Warnick et al. (1985). Otherwise, protein bands interpreted as apo B in the supernatant could represent other proteins.

Next to the protein band indicative of apo B–48, a second and fainter one of ≈ 330 kDa was also visible in both samples, not necessarily corresponding to apoproteins known in the dog. Apo B–100 would be expected to have a higher molecular weight of ≈ 500 kDa (Xenoulis and Steiner, 2010; Mahley and Weisgraber, 1974).

A strong protein band of ≈ 49 kDa was visible in both fractions, and was suspected of representing apo A–IV. As apo A–IV is only known as being associated with HDL in

dogs (Xenoulis and Steiner, 2010), a contamination of the precipitated fraction with HDL is suspected.

Similarly to apo A–IV, a protein band of 23–25 kDa, assumed to represent apo A–I, was observed in both fractions with stronger visibility in the supernatant. Thus apo A–I and apo A–IV were found within the dissolved precipitate, which is in agreement with previous studies involving canine lipoproteins (Barrie et al., 1993a; Asuka et al., 2013), where traces of contamination were found in the precipitate. As an explanation, small amounts of apo A–I could still have been attached to the precipitate or enclosed in its complex, despite washing the precipitated fractions before dissolving them. Or it may indicate that certain lipoproteins containing apo A–I precipitate together with canine apo B–containing lipoproteins, represented by CMs, VLDLs, and LDLs (Xenoulis and Steiner, 2010). A third possibility could be that some canine LDLs possess both apo B and apo A–I, however this property has only been investigated in humans, and showed LDL to have apo B–100 and certain amounts of apo A–I and apo E (Collins et al., 2010).

A faint protein band of 33–35 kDa was seen in both fractions, appearing slightly stronger in the supernatant. As apo E is associated with VLDLs and HDLs (Xenoulis and Steiner, 2010), this protein band probably represents apo E.

Furthermore, a protein band of 61–63 kDa, suspected to be albumin, appeared almost equally strong in both fractions and matrices. This suggests that albumin can also be complexed by heparin manganese chloride which was reported previously for human lipoproteins (Warnick and Albers, 1978).

Protein bands indicative of apo C (7–9 kDa) were not apparent in either fraction, as expected, due to previous dialysis against 10 kDa–sized filters.

Regarding the comparison of plasma and serum, a difference between suspected apo A–I and apo A–IV protein bands occurred, indicating matrix–associated differences. This could possibly be due to the use of lithium heparin plasma, adding additional heparin to the precipitation protocol. Alternatively, heparin could have activated the lipoprotein lipase and modified the lipoprotein structures in the tube, as already hypothesized by others (Moffatt and Stamford, 2005).

Generally, numerous protein bands were seen in both fractions, revealing difficulties in lipoprotein separation and identification of apoproteins. Moreover, the lipid composition of precipitated lipoproteins may differ from that of analogous lipoproteins obtained by flotation techniques. Therefore, precipitation with heparin manganese chloride may be generally effective for a rough and time–saving separation of lipoproteins but may not accurately separate canine LDLs from HDLs. Further, potential alterations of lipid structures render this technique unsuitable for subsequent lipidomic analyses.

4.1.3 Other Lipoprotein Separation Techniques

The traditional approaches such as ultracentrifugation and precipitation did not result in satisfactory separation of canine lipoproteins. Separation according to electrophoretic mobilities has so far been most successful according to Pasquini et al. (2008), however its suitability for subsequent lipidomic analyses is doubtful.

As a different approach, several studies with canine lipoproteins have already used fast

protein liquid chromatography (FPLC) for separation of lipoproteins into three fractions: VLDL, LDL and HDL, accelerating the process of identifying apoproteins (Jericó et al., 2009; Bailhache et al., 2003). It has also been also reported that gel permeation–high performance liquid chromatography (GP–HPLC) is an efficient method in separating canine lipoproteins by size (Mizutani et al., 2010), however GP–HPLC was compared with the ultracentrifugation–precipitation technique and did not achieve better results (Asuka et al., 2013).

Nevertheless, using FPLC in combination with tandem mass spectrometry (MS/MS) to study lipoprotein fractions separated according to their (apo–) proteins would represent an ideal combination and has been achieved in human lipoproteins in a pioneering study by Wiesner et al. (2009). A similar approach with canine lipoproteins should be evaluated for future studies.

4.2 Lipidomics

4.2.1 Lipid Distribution

Fatty Acyls

When compared with all identified lipids (561 identified lipids), fatty acyls (FAs) account for the most diverse lipid category and the specific FAs identified differ greatly between serum and plasma. This is probably due to FAs being a very diverse group of molecules that only have a repeating series of methylene groups in common, (Li et al., 2014). Further, the first subclass of fatty acyls in the canine lipidome ("fatty acids and conjugates") is most diversified, constituting half of all fatty acyls in plasma and serum, similar to what has been found human studies (Fahy et al., 2005).

The predominant fatty acids in animals have been reported to be C_{16} (palmitic acid) and C_{18} species, such as stearic (saturated), oleic (mono–unsaturated) and linoleic acids (poly–unsaturated). Unsaturated fatty acids consist of one (mono–) or more (poly–) unsaturated double bonds that are prone to oxidation. Due to biosynthesis from C_2 units, most fatty acids have been reported to possess an even number of carbon atoms (Voet et al., 2012). In our study, we found FAs with almost any number of carbon atoms, varying from C_5 to C_{42}, with no definite predominance of even–numbered fatty acyls identified.

However, we did find an oelic acid (appendix A.1, FA01 No 48), the number one constituent of human plasma (Quehenberger et al., 2010). Palmitic acid, the second most commonly measured lipid in human plasma (Quehenberger et al., 2010), was not found in its original form in our study, but as a dihydroxy-palmitic-acid instead (appendix A.1, FA01 No 74).

Two polyunsaturated fatty acids (PUFA) were further found to be most abundant in the human plasma lipidome: linoleic acid and arachidonic acid (Quehenberger et al., 2010). Of these, only linoleic acid was found in our study, albeit in a slightly different chemical structure. As for arachidonic acids, only derivates were found in this study.

Of the major fatty acids found in human plasma, comprising about 78% of all free fatty acids in the circulation (Quehenberger et al., 2010), none was found in identical form in the canine lipidome. Although free fatty acids represent only a small fraction of all fatty acids, they are highly active lipids. Their low identification rate in our study may be due to the analytical procedures used, as free fatty acids are better extracted by different lipid extraction techniques followed by gas chromatography instead of by liquid chromatography. Further, adipose tissue is the main source of free fatty acids in plasma (Quehenberger et al., 2010) and the distribution of free fatty acids is potentially related to dietary factors, which differ between humans and dogs. Additionally, the non–occurence of major human lipids in the canine lipidome could be due to susceptibility of PUFAs to oxidation processes (Watson, 2006), despite the addition of an antioxidant (BHT) during the analytical procedures. Especially if looking for lipids involved in atherosclerotic processes, the prevention of further oxidation is essential, as oxidation may continue indefinitely (Malle et al., 2006). As we did not include the identification of oxidative lipids, we missed these lipids in our study.

An important lipid class is the eicosanoids, involved in inflammatory processes and associated with atherosclerosis, where they often function as signaling molecules (Fahy et al., 2005). They are further found to play an important role on cholesterol regulation and seem therefore promising in the therapy of high plasma LDL–cholesterol levels as proposed by Demetz et al. (2014). In our study, eicosanoids were represented by eight identified lipids in plasma and 15 in serum. In comparison, 143 metabolites were found associated with the eicosanoid pathway in the study of Quehenberger et al. (2010), although not all of them were categorized as eicosanoids. Two subclasses of eicosanoids were specifically found to be related to oxygenic processes: hydroxy / hydroperoxy–eicosatetraenoic acids and isoprostanes. In our study, we found five of these lipids. Especially F2-isoprostanes were mentioned as risk factors, which are prostaglandin–like compounds derived from the peroxidation of arachidonic acid (Morrow, 2005). Further, one of the prostaglandins found in this study (appendix A.1, FA03 No 140) was previously reported to be associated with atherosclerosis (Berliner et al., 2009).

Glycerophospholipids

In our study, glycerophospholipids (GPs) were the second largest category of all identified lipids. The majority of all GPs identified in our study are glycerophosphocholines (PCs) and glycerophosphoethanolamines (PEs), similar to those found in human studies (Quehenberger et al., 2010).

As GPs are most widely known as key components of cell membranes, e.g. in lipoproteins, and are expected to represent a high percentage of lipids in the blood. Especially PCs, PEs and PIs (Fahy et al., 2005; Li et al., 2014) represent the most numerous membrane lipids with 50% of all PCs, lysophosphatidylcholines (LPCs) and PEs to be contained within HDLs (Gallego et al., 2010). This percentage is assumed to be even higher in dogs due to their different amount and composition of their HDLs.

Lysophosphatidylcholines (LPCs) are characterized as having a single carbon chain (e.g. 0:0/16:0) and a polar head group, derived from PCs through the action of phospho-

lipase A_2. Due to their structure, they are more hydrophilic than similar phospholipids (Fahy et al., 2005). Specifically, LPC 16:0 was reported to be enriched in human HDL, whereas LPC 18:0 was predominantly associated with VLDL and LDL in humans (Wiesner et al., 2009). Further LPCs have been reported to be a major component of oxidized LDLs in humans and therefore implicated as important factors in their atherogenic activity (Schmitz and Ruebsaamen, 2010). This is in agreement with other studies, where LPCs have been found to generate inflammatory effects, enhancing the process of atherosclerosis (Kita et al., 1999). In this study, we identified 17 LPCs in total, of which at least two (LPC 18:0 and LPC 18:1) have been described as inducing reactive oxygen species in aortic endothelial cells (Li et al., 2016).

Further, we identified one oxidized glycerophosphocholine (oxPC 16:0/9:0(CHO)) despite not focusing on oxidized lipids in our identification program. Oxidized lipids were previously associated with the formation of foam cells in atherosclerotic lesions in mice and humans (Podrez et al., 2002; Tsimikas et al., 2004; Watson, 2006). Therefore, they would represent an interesting group of lipids for future studies regarding atherosclerotic processes in the dog. However, a different lipid extraction protocol would be needed in that case, as GPs are a more hydrophilic group of lipids and our lipid extraction protocol with following research on the organic phase focused on more hydrophobic lipids.

Sterol Lipids

We did find four main classes of sterol lipids (STs) in our study, including sterols (mainly cholesterol and cholesterol esters) as the most numerous group of identified lipids. They have long been mentioned as being involved in human cardiovascular diseases if appearing at highly elevated levels (Hu et al., 2009; Han et al., 2007). Cholesterol is also the most abundant sterol in animals in terms of lipid concentrations, as verified by other studies (Li et al., 2014), which is similar to humans, where cholesterol and cholesterol esters were found to be among the most abundant metabolites in serum (Psychogios et al., 2011) and plasma (Quehenberger et al., 2010). As no internal standards were included in our study, no comparison of lipid concentrations to other studies can be carried out.

The occurrence of cholesterol and cholesteryl esters (CEs) in the blood is closely linked to lipoproteins, as explained in the introduction. Neutral CEs represent the most abundant lipid class in humans with CE 18:2 accounting for over half of it, followed by eicosapentaeonic acid (CE 20:4) (Quehenberger et al., 2010). Both of them were found in our study, among five other CEs. Three of these CEs (18:3, CE 20:4, CE 20:5) were also found to be associated with fatal cardiovascular outcome in human studies (Tarasov et al., 2014; Stegemann et al., 2011).

Another CE identified in our study (CE18:1) was investigated in a study by Degirolamo et al. (2009), revealing human LDLs enriched in mono–unsaturated cholesteryl oleate (CE 18:1) to be more active in binding to arterial proteoglycans than other LDLs, favoring the formation of atherosclerotic lesions. This underlines the importance of lipidomic analyses of lipoprotein compositions rather than focusing on the concentration of lipoproteins only.

Glycerolipids

In humans, glycerolipids (GLs) represent a high proportion of the total lipids in plasma (McAnoy et al., 2005). This is expected to be similar in dogs, as triglycerides are one of the most numerous lipids occurring in CMs and VLDLs. However, as no quantitative study was performed, we can only compare the identification of specific lipids and not their concentration within the blood.

Distinguishing between isomeric species was a challenge. This is due to the varying number of FAs that are esterified to the hydroxyl groups attached to the glycerol backbone. As those FAs can permute at various positions, stereochemical composites (isomers) are formed (Fahy et al., 2005). Therefore, similar to human studies (Fahy et al., 2005), we found the majority of GLs as isomeric forms with each diglyceride having two isomeric species and each triglyceride, with its three different radyl groups, resulting in six possible isomers.

In most studies, natural FAs found in TGs from plants or animals have been reported to consist of long hydrocarbon chains with an even number of carbon atoms (Hu et al., 2009). This is in line with our results; however we only identified nine TGs in our study, of which seven were even–numbered. Within the blood, TGs are distributed between CMs and VLDLs and are therefore highly dependent on food intake (Quehenberger and Dennis, 2011), potentially differing between humans and dogs.

Considering all isobaric species, over 200 individual molecular species of TGs were detected in human plasma with TG 50:2, 52:2 and 52:4 being the most abundant GLs (Quehenberger et al., 2010) of which only TG 52:3 was identified in our study and further in plasma only.

DGs were present at substantially lower levels in human blood than TGs, with 1,2–DGs being about three times more abundant than 1,3–DGs (Quehenberger et al., 2010). In our study, we found 1,2–DGs to be the vast majority of DGs identified, however we did not quantitatively assess them.

Sphingolipids

Sphingolipids (SPs) are usually found in cell membranes and composed of a sphingoid backbone with various fatty acids attached to it (Watson, 2006). They are categorized into sphingoid bases, ceramides (sphingoid bases with amide–linked fatty acids), phosphosphingolipids, including sphingomyelins, and glycosphingolipids such as cerebrosides and gangliosides (Fahy et al., 2005). Of these, ceramides (Cer) and sphingomyelins (SMs) are biologically most important (Hu et al., 2009).

In our study, we identified 39 different SPs, of which Cers were the most numerous class, followed by SMs. This distribution differs from that in human plasma, where SMs accounted for the largest fraction of SPs (Quehenberger et al., 2010). However, as we used a different lipid extraction method than Quehenberger et al. (2010) and only identified a small number of SPs, distribution may vary.

SPs, especially Cers and SMs, have been reported to influence the atherogenic process by affecting the lipoprotein metabolism and are therefore associated with the development of atherosclerotic lesions (Bismuth et al., 2008; Alewijnse and Peters, 2008). Cers were

also found in a 10 to 50–fold higher content in LDLs within an atherosclerotic lesion in human patients than in plasma LDLs (Schissel et al., 1996). However, the interaction of Cers, oxidized LDLs, inflammatory cytokines and endothelial cells is not entirely clear so far (Bismuth et al., 2008).

Further, ratios of several Cers have been associated with fatal cardiovascular outcome in a human study, including the ratio of Cer(d18:1/24:1) to Cer(d18:1/24:0) (Tarasov et al., 2014), both of which occurred in our study.

Sphingomyelins (SMs) consist of ceramides with either an additional phosphocholine or phosphoethanolamine head group. They are found most frequently as membrane lipids in myelin sheaths (Voet et al., 2012) but also contribute to the major lipoprotein membrane lipids in the human blood (Wiesner et al., 2009). This explains why the different SMs are found in such a high number within canine blood.

To summarize, CEs, PCs including LPCs, Cers and SMs are the main interesting lipid classes associated with atherosclerosis and were also significantly altered comparing atherosclerotic plaques to plasma samples (Stegemann et al., 2011).

Regarding the distribution of SMs within lipoproteins, different studies revealed different results. A study by Li et al. (2014) revealed SMs to account for half of the lipids contributing to the total HDL mass in humans, together with phospholipids, cholesteryl esters, TGs and free cholesterol. In contrast, the study of Wiesner et al. (2009) reported that SMs only account for 5–10% of all lipids in HDLs, whereas LDLs contain the largest amount of the plasma SMs.

Prenol Lipids

Prenol lipids (PRs) and their main group of isoprenoids are synthesized from five carbon precursors (C5), therefore they consist of multiplicities of this unit such as C10 isoprenoids, C15 and so on (Fahy et al., 2005).

We identified a total number of 37 prenol lipids in our study, the majority being isoprenoids (34 lipids) of which most consisted of C15 molecules.

Polyketides

Most of the polyketides identified in our study were flavonoids, mostly known to originate from plants or microbial sources (Fahy et al., 2005), and therefore assumed to arise from the diet that our dogs had received during the study. Furthermore, the occurrence of flavonoids may prove that some amphiphilic lipids were recovered in the organic phase of our lipid extraction.

4.2.2 Plasma vs. Serum

Amounts of lipids identified in both matrices were expected to differ only slightly between plasma and serum, with plasma being obtained from whole blood in the presence of anticoagulants and serum being harvested after a clotting process. However, many of the 561 lipids identified in total were only found in either plasma or serum in our study,

similar to humans were the lipidomic profiles of the two matrices were found to differ as well (Ishikawa et al., 2013).

In our study, comparing the 246 lipids identified in both plasma and serum, we did find eleven lipids to be significantly altered in their mean abundances between plasma and serum, analyzed by a paired Student's *t*–test and corrected with a false discovery rate (fdr). However, usage of fdr gives 5% of significant results by default. As we have $\approx 4.5\%$ significant results, log2 ratios of mean abundances in plasma versus serum were also taken into consideration.

Lipids with ratios >2 or <–2 were interpreted to represent important differences, resulting in the three lipids further discussed here: 14–keto pentadecanoic acid, an oxidative fatty acid; 1–(2,5–dihydro–6,8–dihydroxy–3–methyl–1–benzoxepin–7–yl)–3–phenyl–1–propanone, a flavonoid categorized into the polyketides; and (+)–Blennin D, an isoprenoid categorized into the prenol lipids.

Oxidative fatty acids were previously reported to be associated with the clotting process of serum, as they may be derived from arachidonic acid, which in turn is released from membrane phospholipids in platelets during blood coagulation (Ishikawa et al., 2013).

But the ratio of the oxo fatty acid found here is enhanced in plasma compared to serum. Hence, there are probably other reasons than the clotting process for this specific lipid to differ greatly between plasma and serum.

Similarly, eicosanoids are thought to differ greatly between serum and plasma, due to release from activated platelets, as they are derivates of arachidonic acid (Ishikawa et al., 2014). However, no significant eicosanoids were found in our study, probably due to their distribution within the matrices. Of 19 different eicosanoids found within the dog, only four were found in both matrices and could therefore be compared. Eleven eicosanoids were found in serum exclusively, supporting the theory of enhanced eicosanoids in serum due to the clotting process (Liu et al., 2010).

Similar to eicosanoids, 21 sphingolipids were identified in serum only, whereas eight occurred in plasma only and ten were found in both matrices. This supports serum as the matrix of choice for eicosanoids and sphingolipids. Further, sphingolipids were also reported as potential biomarkers for atherosclerotic processes (Liu et al., 2010), which is in agreement to other studies, revealing serum to include more potential biomarkers than plasma (Yu et al., 2011).

In addition, and possibly related to the clotting process, may be the second lipid, a flavonoid, which was highly enhanced in plasma. Nothing is known so far on that specific lipid, but flavonoids in general are widely distributed plant polyphenols (Dangles et al., 2000). Due to their ability to inhibit several enzymes, they potentially act as antioxidants, playing an important role in the protective mechanisms against cardiovascular diseases (Middleton Jr and Kandaswami, 1994). Further, as flavonoids are reported to inhibit the release of thromboxane, their higher concentration in plasma than serum is potentially related to the clotting process (Vita, 2005).

The last lipid found to be highly enhanced in plasma is an isoprenoid, categorized into the main class of sesquiterpenes. While not much is known concerning this lipid specifically, sesquiterpenes have already been investigated regarding their interactions with plasma and serum in other studies. The results indicate a different protein binding

activity of various sesquiterpenes to plasma or serum albumin (Wagner et al., 2004). Thus, a possible high bondage of the lipid found here with albumin is assumed, and possibly associated with a lower amount of this sesquiterpene in serum after the clotting process.

In summary, our study showed that if the same analytical procedures are used, plasma and serum only differ slightly in the amount of lipids measured in both matrices. However, as no multiple measurements were performed, the reproducibility of these results cannot be commented. Further, when targeting a special category or individual lipids, the choice of matrix is crucial, as many lipids are only found in either plasma or serum. Additionally, serum results should be viewed with caution, as they are altered due to the clotting process. Plasma on the other hand represents the original properties of the blood, only potentially modified by added lithium heparin.

Regarding the different anticoagulants, no general consensus exists in the lipidomic community. The difference between anticoagulants lies in their mechanisms to prevent the clotting formation with citrate and EDTA chelating Ca^{2+}, while heparin binds to enzymes responsible for the coagulation cascade (Gonzalez-Covarrubias et al., 2013). Since lipid extraction should eliminate most of the anticoagulant salts before LC–MS analysis (Gonzalez-Covarrubias et al., 2013), possible interactions with lipids must occur during initial contact. As the use of lithium heparin has been shown to result in slightly more identified lipid species than with EDTA or sodium citrate, some authors recommend lithium heparin as a suitable anticoagulant (Vuckovic, 2012; Pereira et al., 2010).

4.2.3 Prednisolone Treatment

The effect of hyperadrenocorticism and enhanced levels of glucocorticoids (GCs) upon lipoprotein compositions in dogs has been studied by various research teams (Barrie et al., 1993b; Mahley et al., 1974; Ling et al., 1979; Bauer, 2004; Xenoulis and Steiner, 2010). Regarding the effects of GCs on the lipid metabolism, increased lipolysis, lipid oxidation and insulin–antagonistic effects have already been mentioned, resulting in enhanced free fatty acids with enhanced levels of TGs and eventually TG–rich lipoproteins such as VLDLs (Barrie et al., 1993b). Increased plasma cholesterol concentrations and partly increased LDL–cholesterol concentrations or enhanced levels of LDLs and VLDLs are also known to occur after GC treatment (Barrie et al., 1993b; Bauer, 2004). As in the present study, prednisolone treatment was only short term, so no long–term consequences as seen with hyperadrenocorticism were expected. However, as we used a high dose prednisolone treatment, some differences were still assumed to be observed afterwards.

Beside the immediate effect of GCs on the lipid metabolism by lipolysis, a rapid anti–inflammatory response is promoted by arachidonic acid derivates. This may occur through an altered membrane lipid metabolism caused by GCs. Further, as the enzyme COX–2 is inhibited by GCs, synthesis of pro–inflammatory prostaglandins is suppressed (Malcher-Lopes et al., 2008).

Canine Plasma

Ten lipids were significantly altered after prednisolone treatment in plasma. However, as a false discovery rate was used as mentioned above, log2 ratios of mean abundances were taken into consideration. If the log2 ratio was >2 or <–2, lipids were assumed to be greatly altered after prednisolone treatment. Therefore only six of the ten significantly altered lipids are discussed here at length.

All of these six lipids are fatty acids, which seems plausible, as FAs represent $\approx 45\%$ of all lipids identified in canine plasma. Further, five of the six FAs discussed here were increased after prednisolone treatment, possibly through lipolysis, whereas only one FA was significantly decreased after prednisolone.

The lipid most increased after prednisolone treatment was Docosatriynoic acid, an unsaturated fatty acid with a log2 ratio of 3.9. Docosatriynoic acid (22:3 δ10 ω6) is an acetylenic acid, where δ designates the position of the first double bond, counted from the carboxyl end of the fatty acid, and ω designates the position of the last double bond, counted from the terminal carbon of the fatty acid (Sams, 1981). A possible explanation for the high amount of this lipid after prednisolone treatment could be the inhibition of prostaglandin synthesis through COX–2 inhibition achieved by GCs. This may be due to prostaglandins being formed by enzymatic oxygenation of certain essential polyunsaturated fatty acids (PUFAs) (Sams, 1981). As the lipid discussed here is also a PUFA, it may be enhanced because it was not synthesized into prostaglandin. However, many lipids are PUFAs and only some of them are included in the prostaglandin pathway. Another study revealed acetylenic acids to inhibit the prostaglandin synthesis from certain unsaturated fatty acids in cows. The most potent inhibitors were octadeca–6,9,12–triynoic acid and eicosa–8,11,14–triynoic acid, however docosa–10,13,16–triynoic acid was also mentioned as being inhibitory, although less potent (Goetz et al., 1976). Hence, release of 10,13,16–docosatriynoic acid could be triggered by prednisolone treatment to further inhibit prostaglandin synthesis.

Likewise, DPA (docosapentaenoic acid, 22:5n–3) was threefold increased after treatment, an ω3 PUFA with a log2 ratio of 3.2 after prednisolone treatment. This n–3 DPA has been mentioned several times in studies, mostly as a fish–derived fatty acid. It is a metabolite of eicosapentaenoic acid (EPA, 20:5n–3) and an intermediary product between EPA and docosahexaenoic acid (DHA, 22:6n–3), the end product of the essential fatty acid metabolism. It has been suggested that n–3 DPA has beneficial effects regarding cardiovascular diseases (Kaur et al., 2011; Rissanen et al., 2000). Further it is metabolized by lipoxygenase in platelets and also inhibits aggregation of platelets, at least in rabbits. Furthermore, n–3 DPA has been shown to possess a tenfold greater endothelial cell migration ability than EPA, important in wound healing processes (Kaur et al., 2011). In regard to inflammatory processes, both n–6 PUFA such as arachidonic acids, the precursors of inflammatory eicosanoids, and n–3 PUFAs EPA and DHA are interesting. EPA and DHA inhibit the arachidonic acid metabolism and act, in the end, anti–inflammatorily or give rise to mediators that are less inflammatory. Furthermore, they have anti–inflammatory effects on leukocyte chemotaxis and cytokine production (Calder, 2008; Groeger et al., 2010). n–3 DPA was also described to be responsible for cholesterol–lowering activity

together with DHA, therefore lowering the risk of developing atherosclerosis (Chen et al., 2012). To summarize, the reason for the high occurrence of DPA after prednisolone treatment may be explained by its being an intermediate product between EPA and DHA, involved in the anti–inflammatory response.

Another lipid is 3–hydroxy–hexadecanoic acid with a log2 ratio after prednisolone treatment of 2.3. Not much is known of this hydroxy fatty acid, a derivate of palmitic acid, but it has been associated with a certain antioxidant activity, together with 3–hydroxytetradecanoic acid and palmitic acid (Tel et al., 2010).

Having the same log2 ratio of 2.3 after prednisolone treatment, 9R–hydroxy–octadecanoic (9–HODE) is an oxidation product of linoleic acid and regulated through the LOX pathway, whilst HETEs are derived from arachidonic acids. HODEs, more specifically 9– and 13–HODE, have been detected as the most abundant oxidation products in atherosclerotic plaques, together with 15– and 11–HETEs, although in smaller amounts. Further, they have also been identified in increased amounts in the LDLs of these atherosclerotic plaques (Kuhn et al., 1992). In humans, almost 50% of the PUFAs in LDLs are made of linoleic acid, which is susceptible to oxidation. Therefore, HODEs and specific HETEs may account for biomarkers in plasma representing the degree of oxidative damage (Jira et al., 1998). Some of the HETEs also activate chemotaxis of neutrophils (Goetzl et al., 1980), whilst HODEs stimulate foam cell formation through upregulation of peroxisome proliferator activated receptors and resulting monocyte–macrophage differentiation (Tontonoz et al., 1998).

3R–hydroxyoctadecanoic acid (3–HODE) has a slightly lower log2 ratio of 2.1 after prednisolone treatment and has also been associated but not specifically identified within atherosclerotic plaques. However, the occurrence of HODEs may be triggered in some way by prednisolone, as glucocorticoids enhance the stress level within the body, potentially stimulating oxidative stress processes.

The only lipid found to be drastically decreased after prednisolone treatment was hydroxyphthioceranic acid (C39), a long–chain branched fatty acid with a log2 ratio of –3.2. This lipid has so far mostly been associated with Mycobacterium tuberculosis as a key component of its cell–wall (Sirakova et al., 2001). Reasons for the occurrence of such a lipid in the blood of dogs can only be speculated upon. However, as it was found in all dogs, it potentially has further functions than being a cell component of M. tuberculosis.

Four other lipids were also found to be significantly altered after prednisolone treatment in plasma but displayed ratios of <2 or >-2, therefore the relevance of these lipids may be decreased as potentially false positive results. 8,11–eicosadiynoic acid is one of them, an unsaturated fatty acid with a log2 ratio of 1.7 after prednisolone treatment and known to inhibit the eicosanoid biosynthesis at several stages (Laposata et al., 1987). An upregulation under prednisolone treatment seems therefore plausible as eicosanoids are associated with inflammation in the body.

PE(P–16:0/22:4), a glycerophosphoethanolamine with a log2 ratio of 0.6 after prednisolone treatment is reported to consist of two parts: one plasmalogen chain 16:0 and one adrenic acid. Plasmalogen 16:0 is derived from animal fat, liver and kidney, whereas adrenic acid is mostly derived from animal fats (Wishart et al., 2013). Previous studies reported plasmalogen to function as an antioxidant, also for LDLs to prevent oxidation

(Vance, 1990).

The last two significantly altered lipids are decreased after prednisolone treatment, MG(18:1(11E)/0:0/0:0) with a log2 ratio of –0.6 and 11Z–octadecen–9–ynoic with a log2 ratio of –0.8. As MGs are derived from DGs and TGs due to lipolysis, the decreased levels after prednisolone are surprising. Similarly 11Z–octadecen–9–ynoic, a PUFA, could not be directly linked to glucocorticoids.

Canine Serum

Of the three lipids tested to be significantly altered in serum after prednisolone treatment, none of them showed log2 ratios of >2 or <–2. Hence, they will not be thoroughly discussed here, with the exception of 9R–hydroxy–octadecanoic acid. This lipid was found to be enhanced in plasma as well as serum after prednisolone treatment, however the log2 ratio in serum was only 0.9 compared to plasma with 2.3 As both serum and plasma were drawn at the same time and matrices underwent the same analytical steps, prednisolone treatment seems to show a greater impact on the relative abundances of this lipid in plasma than in serum. This may be either due to matrix–associated differences affected by the clotting process, or due to inaccuracy during lipid measurements. Generally, as plasma produced a wider range of statistically significant results and is assumed to represent the conditions unaltered as in vivo without the clotting process, plasma seems more suitable for further lipidomic studies.

The other two lipids found in canine serum to be significantly altered after prednisolone treatment were adrenic acid and PE(20:4/20:0). Adrenic acid, a PUFA, was enhanced after prednisolone treatment and can be found in the adrenal gland, brain, kidney, and vasculature (Mann et al., 1986). Similar to arachidonic acid, it is metabolized by COX, LOX and CYP450 enzymes to dihomo eicosanoids (Kopf et al., 2010). Therefore, it seems possible that prednisolone enhances its amount in the blood due to inhibiting further enzyme reactions.

The decrease of PE(20:4/20:0) on the other hand cannot be readily explained; however it consists of one chain of arachidonic acid and one chain of arachidic acid (**?**), which may associate it with the effects of GCs.

4.3 Limitations of the Study

4.3.1 Lipoprotein Separation

Both protocols A and B were made of blood from the same dog, therefore no comparison with other dogs or mean values of different dogs were possible and the power of the study is low. Nevertheless, our results are sufficient to demonstrate the altering density ranges of canine to human lipoproteins and verify published studies. Due to the overlapping density ranges in the dog, no further investigations were performed using ultracentrifugation.

A possible limitation of protocol B could be the usage of lithium heparin instead of EDTA plasma, as was used in previous protocols, potentially enhancing the heparin concentration during precipitation. However, other studies with differing compositions of

heparin manganese chloride did not achieve an accurate separation either (Asuka et al., 2013). Further, we only estimated molecular weights based on manual measurements on SDS–PAGEs. Apoproteins were assumed based on estimated molecular weights and not identified by further techniques such as GP–HPLC. However, as major apoproteins of canine LDL and HDL differ greatly in their molecular weights, our protocols seemed sufficient for our goals.

4.3.2 Lipidomics

This study was a pilot study to investigate the lipidome of healthy and prednisolone treated dogs. As no other studies on the lipidome of dogs are known so far, comparisons could not be made.

Further, using LC–MS/MS as done in this study, a tighter spectrum of lipids can be detected and identified, although potentially missing others. But even in this study, using LC prior to MS analyses, several lipids with overlapping mass to charge (m/z) ratios were detected, enhancing the difficulty of accurate lipid identification. Moreover, as only one lipid extraction protocol was used in this study, not all lipids are equally extracted into the organic phase due to differing polarities. With MTBE–based lipid extraction by Matyash et al. (2008), the main lipid classes were believed to be extracted with similar high recoveries as achieved by the classic lipid extraction (Folch et al., 1957). However, semipolar and especially polar compounds (e.g. phospholipids) are probably retrieved in lower amounts than they actually occur due to the biphasic nature of lipid–lipid extractions. Finally, only non–polar lipid species are presented at a high level through our LC–MS approach, whereas for polar lipids a GC–MS system probably would have been more suitable (Teo et al., 2015).

Regarding quantitative measurements, this study was only semiquantitative, as lipid extracts were not spiked with internal standards. These standards would typically compromise isotopically labeled or non–endogenous odd–chain lipids, possessing similar characteristics as the lipid species wished to target (Teo et al., 2015). However, as the same procedure was used on all samples and plasma and serum were taken from the same dogs at the same time, comparison between samples still results in reliable semiquantitative findings.

Also, no repeated measurements of the same samples were done, which would enhance reliable lipid identification and quantification. Correction for multiple testing was performed using a false discovery rate (fdr); therefore if results were $<5\%$, false positive results may have occurred as discussed above. Hence log2 ratios should be taken into consideration. Further, statistical statements including p–values from semiquantitative mean abundances can be deceptive due to the low number of dogs compared, the effect of age and gender of the animals and the high sensitivity of mass spectrometric measurement techniques to minor differences in sample handling or analytical procedures.

Looking at the identified and statistically significant lipids gives us points of interests, however our biological knowledge of these lipids is small and mostly related to the lipid class rather than the individual identified lipids. After detecting significantly altered lipids, the next challenge is to discern whether they represent actual alterations caused

by the given treatment.

Although great advances in the field of mass spectrometric researches have been achieved and it has proved to be a valuable tool for lipid analysis, there is always potential for development. Minor components or oxidized lipids often still remain undetected due to ion suppression of low–abundance species by high–abundance species, although detection is better with chromatographic separation in advance (Hu et al., 2009; Teo et al., 2015). In this study, no oxidized lipids were included in the lipid identification process but should be focused on in a future study due to their role in atherosclerotic plaques.

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Appendix A

Supplemental Material

A.1 Canine Lipidome

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
	Fatty Acyls (FAs)						
1	FA00	Other Fatty Acyls		Serratamic acid	N–(3S–hydroxydecanoyl)–L–serine	276	P
2	FA01	Fatty Acids and Conjugates	Branched fatty acids	18:1(12E)(11Me)	11–methyl–12E–octadecenoic acid	314	P
3	FA01	" "	" "	18:1(7Z)(17Me)	17–methyl–7Z–octadecenoic acid	314	P / S
4	FA01	" "	" "	Hydroxyphthioceranic acid (C31)	2S,4S,6S,8R,10R–pentamethyl–11–hydroxy–hexacosanoic acid	483	S
5	FA01	" "	" "	Hydroxyphthioceranic acid (C34)	2S,4S,6S,8R,10R,12R–hexamethyl–13–hydroxy–octacosanoic acid	526	P / S
6	FA01	" "	" "	Hydroxyphthioceranic acid (C36)	2S,4S,6S,8R,10R,12R–hexamethyl–13–hydroxy–triacontanoic acid	571	P / S
7	FA01	" "	" "	Hydroxyphthioceranic acid (C39)	2S,4S,6S,8S,10R,12R,14R–heptamethyl–15–hydroxy–dotriacontanoic acid	613	P / S
8	FA01	" "	" "	Hydroxyphthioceranic acid (C40)	2S,4S,6S,8S,10R,12R,14R,16R–octamethyl–17–hydroxy–dotriacontanoic acid	627 / 610	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
9	FA01	" "	" "	Hydroxyphthioceranic acid (C42)	2S,4S,6S,8S,10R,12R,14R,16R–octamethyl–17–hydroxy–tetratriacontanoic acid	655	P / S
10	FA01	" "	" "	Hydroxyphthioceranic acid (C37)	2S,4S,6S,8S,10R,12R,14R–heptamethyl–15–hydroxy–triacontanoic acid	585	S
11	FA01	" "	" "	Mycocerosic acid (C28)	2R,4R,6R–trimethyl–pentacosanoicacid	442	P / S
12	FA01	" "	" "	Mycolipenic acid (C29)	2,4S,6S–trimethyl–2E–hexacosenoicacid	454	S
13	FA01	" "	" "		22–methyl–tricosanoic acid	386	P / S
14	FA01	" "	" "		2–methyl–hexadecanoic acid	253 / 303	P / S
15	FA01	" "	" "		2–methyl–tetradecanoic acid	225 / 260	P / S
16	FA01	" "	" "		2oxo–3R–methyl–pentanoic acid	113	P
17	FA01	" "	" "	-	4–methyl–octadecanoic acid	316	P / S
18	FA01	Fatty Acids and Conjugates	Unsaturated fatty acids	–	10,13,16–Docosatriynoic acid	351	P
19	FA01	" "	" "	–	10E–heptadecen–8–ynoic acid	282 / 297	P / S
20	FA01	" "	" "	–	10–hydroxy–2Z,8Z–Decadiene–4,6–diynoic acid	163 / 213	P / S
21	FA01	" "	" "	–	10–Hydroxy–3,7–dimethyl–2E,6E–decadienoic acid	195	P / S
22	FA01	" "	" "	–	11Z–octadecen–9–ynoic acid	296 / 279	P / S
23	FA01	" "	" "	–	12,14–Pentacosadiynoic acid	407	P
24	FA01	" "	" "	–	13–hexadecenoic acid	272 / 277	P / S
25	FA01	" "	" "	–	14Z–tricosenoic acid	370	P / S
26	FA01	" "	" "	–	15Z–eicosenoic acid	328	P / S
27	FA01	" "	" "	–	18–nonadecynoic acid	312	P
28	FA01	" "	" "	–	19Z–octacosenoic acid	440	P / S
29	FA01	" "	" "	–	3E–tetradecenoic acid	227	P
30	FA01	" "	" "	–	3–heptadecenoic acid	269	P
31	FA01	" "	" "	–	4,9–octadecadiynoic acid	277	P
32	FA01	" "	" "	–	4E–Undecene–6,8,10–triynoic acid	205	S
33	FA01	" "	" "	–	4–tridecynoic acid	193 / 228	P / S
34	FA01	" "	" "	–	4–undecynoic acid	183	P
35	FA01	" "	" "	–	6,9–heptadecadiynoic acid	280	S
36	FA01	" "	" "	–	8,11–Eicosadiynoic acid	287	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
37	FA01	" "	" "	–	9,12,15–Eicosatriynoic acid	301	P
38	FA01	" "	" "	–	9–heptadecynoic acid	267 / 284	P / S
39	FA01	" "	" "	–	9–hexacosenoic acid	412	P / S
40	FA01	" "	" "	–	9–hydroxy–7Z–Nonene–3,5–diynoic acid	165	P
41	FA01	" "	" "	17:1(11Z)	11Z–heptadecenoic acid	269	P / S
42	FA01	" "	" "	21:1(7Z)	7Z–heneicosenoic acid	342	P / S
43	FA01	" "	" "	21:3(5Z,14Z,17Z)	5Z,14Z,17Z–heneicosatrienoic acid	338	P / S
44	FA01	" "	" "	29:2(5Z,9Z)	5Z,9Z–nonacosadienoic acid	452	P / S
45	FA01	" "	" "	34:6(16Z,19Z,22Z,25Z,28Z,31Z)	16Z,19Z,22Z,25Z,28Z,31Z–tetratriacontahexaenoic acid	529	P
46	FA01	" "	" "	cis,trans–hepta–2,4,6–trienoic acid	2Z,4E,6–heptatrienoic acid	157	S
47	FA01	" "	" "	Linoelaidic acid	9E,12E–octadecadienoic acid	281 / 298	P / S
48	FA01	" "	" "	Oleic acid(d2)	9Z–octadecenoic acid(d2)	267	P
49	FA01	" "	" "	TrHA	5Z,8Z,11Z,14Z,17Z,20Z–tricosahexaenoic acid	360	P / S
50	FA01	" "	" "	δ2–THA	2E,6Z,9Z,12Z,15Z,18Z,21Z–tetracosaheptaenoic acid	372	P / S
51	FA01	Fatty Acids and Conjugates	Hydroperoxy–fatty acids		5–hydroperoxy–7–[3,5–epidioxy–2–(2–octenyl)–cyclopentyl]–6–heptenoic acid	355	S
52	FA01	" "	" "	–	methyl 15–hydroperoxy–9Z,12Z,16E–octadecatrienoate	307	P / S
53	FA01	Fatty Acids and Conjugates	Hydroxy fatty acids	–	11S–hydroxy–tetradecanoic acid	245 / 267	P / S
54	FA01	" "	" "	–	12–hydroxy–5,8,10–heptadecatrienoic acid	263	S
55	FA01	" "	" "	–	13,14–dihydroxy–docosanoic acid	373	P / S
56	FA01	" "	" "	–	16–hydroxy–9E–hexadecenoic acid	288	P
57	FA01	" "	" "	–	17–hydroxy–heptadecanoic acid	304	P / S
58	FA01	" "	" "	–	19–hydroxy–nonadecanoic acid	297	P / S
59	FA01	" "	" "	–	21–hydroxy–heneicosanoic acid	360	P / S
60	FA01	" "	" "	–	24–hydroxy–tetracosanoic acid	402	P
61	FA01	" "	" "	–	2–hydroxy–10–undecenoic acid	201	P
62	FA01	" "	" "	–	3–hydroxy–hexadecanoic acid	290	P / S
63	FA01	" "	" "	–	3R–hydroxy–eicosanoic acid	311	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
64	FA01	" "	" "	–	3R–hydroxy–pentadecanoic acid	259 / 241	P / S
65	FA01	" "	" "	–	7–hydroxy–10–heptadecen–8–ynoic acid	300	P / S
66	FA01	" "	" "	–	methyl 8–[2–(2–formyl–vinyl)–3–hydroxy–5–oxo–cyclopentyl]–octanoate	311	S
67	FA01	" "	" "	–	methyl 9,12–dihydroxy–13–oxo–10–octadecenoate	360	S
68	FA01	" "	" "	11,12–dihydroxy arachidic acid	11,12–dihydroxy–eicosanoic acid	362	S
69	FA01	" "	" "	2,15–dihydroxy–pentadecylicacid	2,15–dihydroxy–pentadecanoic acid	292	P / S
70	FA01	" "	" "	2–hydroxy behenic	2–hydroxy–docosanoic acid	357 / 374	P / S
71	FA01	" "	" "	2–hydroxy–3–methylhexadecanoic–acid	2–hydroxy–3–methylhexadecanoicacid	304	P / S
72	FA01	" "	" "	2R–hydroxylauric acid	2R–hydroxydodecanoic acid	217	P
73	FA01	" "	" "	3–hydroxy–isoheptanoic acid	3–hydroxy–6–methyl–hexanoic acid	183	P / S
74	FA01	" "	" "	9,16–dihydroxy–palmitic acid	9,16–dihydroxy–hexadecanoic acid	311 / 306	P / S
75	FA01	" "	" "	Axillarenic acid	11,13–dihydroxy–9E–tetracosenoic acid	399	P
76	FA01	" "	" "	Hydroxynervonic acid	2–hydroxy–15Z–tetracosenoic acid	400	P / S
77	FA01	Fatty Acids and Conjugates	Oxo fatty acids	–	12–oxo–5E,8E,10Z–dodecatrienoic acid	247	P
78	FA01	" "	" "	–	18–oxo–nonadecanoic acid	330	S
79	FA01	" "	" "	–	2–oxo–docosanoic acid	372	P / S
80	FA01	" "	" "	–	3–oxo–eicosanoic acid	344 / 309	P / S
81	FA01	" "	" "	–	3–oxo–nonadecanoic acid	330	P
82	FA01	" "	" "	–	4,7–dioxo–octanoic acid	173	P
83	FA01	" "	" "	–	5–oxo–7–octenoic acid	139 / 157	P / S
84	FA01	" "	" "	–	7–oxo–11Z–Tetradecenoic acid	258	S
85	FA01	" "	" "	14–keto pentadecanoic acid	14–oxo–pentadecanoic acid	279 / 257	P / S
86	FA01	" "	" "	3–oxohexacosanoic acid	3–oxohexacosanoic acid	428	S
87	FA01	" "	" "	4–keto pentadecanoic acid	4–oxo–pentadecanoic acid	257	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
88	FA01	" "	" "	7–methyl–3–oxooctanoic acid	7–methyl–3–oxooctanoic acid	190	P
89	FA01	" "	" "	9–Keto heptadecylic acid	9–oxo–heptadecanoic acid	267	P / S
90	FA01	Fatty Acids and Conjugates	Epoxy fatty acids	–	methyl 12,13–epoxy–9,15–octadecadienoate	309	P / S
91	FA01	Fatty Acids and Conjugates	Methoxy fatty acids	–	12,13–dihydroxy–11–methoxy–9–octadecenoic acid	362	P / S
92	FA01	Fatty Acids and Conjugates	Halogenated fatty acids	–	9–hydroxy–10–chloro–octadecanoic acid	352	P
93	FA01	" "	" "	14–fluoro–myristic acid	14–fluoro–tetradecanoic acid	279	P
94	FA01	Fatty Acids and Conjugates	Amino fatty acids	–	11–amino–undecanoic acid	184	S
95	FA01	" "	" "	–	2E,4E,8E,10E–Dodecatetraenedioic acid	223	P / S
96	FA01	" "	" "	–	4–methyl–tridecanedioic acid	259	P / S
97	FA01	" "	" "	–	5–methyl–tetradecanedioic acid	273	P / S
98	FA01	" "	" "	–	9–hydroxy–hexadecan–1,16–dioic acid	320	S
99	FA01	" "	" "	3–hydroxy–sebacic acid	3–hydroxy–decanedioic acid	236	P
100	FA01	" "	" "	Docosanedioic acid	Docosanedioic acid	388	P / S
101	FA01	" "	" "	Hexacosanedioic acid	Hexacosanedioic acid	444 / 427	P / S
102	FA01	" "	" "	Tridecanedioic acid	Tridecanedioic acid	245	P
103	FA01	Fatty Acids and Conjugates	Carbocyclic fatty acids	Majusculoic acid	4S,5S–methylene–11–bromo–8E,10E–tetradecadienoic acid	297	P / S
104	FA01	" "	" "	Oncobic acid	15–(2–cyclopenten–1–yl)–8–pentadecenoic acid	289	P
105	FA01	" "	" "	Sterculynic acid	7–(2–non–8–yn–1–ylcycloprop–1–en–1–yl)heptanoic acid	323	S
106	FA01	Fatty Acids and Conjugates	Dicarboxylic acids	–	10–hydroxy–16–oxo–hexadecanoic acid	304	P / S
107	FA01	" "	" "	–	2E–Decenedioic acid	218	P
108	FA01	" "	" "	–	5–methyl–hexadecanedioic acid	323	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
109	FA01	" "	" "	2,2–dimethyl–succinic acid	2,2–dimethyl–butanedioicÂ acid	129	P / S
110	FA01	" "	" "	3–hydroxy–tetradecanedioic acid	3–hydroxy–tetradecanedioic acid	292 / 297	P / S
111	FA01	" "	" "	Ceriporic acid C	1,10Z–heptadecadiene–2,3R–dicarboxylic acid	370	P / S
112	FA01	" "	" "	Eicosanedioic acid	Eicosanedioic acid	360	P
113	FA01	" "	" "	Suberic acid	1,8–octanedioic acid	157	P
114	FA02	Octadeca–noids	12–oxophytodienoic acid metabo-lites	–	(1R,2R)–3–oxo–2–pentyl–cyclopentanehexanoic acid	269	S
115	FA02	" "	" "	(9R,13R)–10–oxo–11–phytoenoic acid	(9R,13R)–2–oxo–5–pentyl–3–cyclopentene–1–octanoic acid	312	S
116	FA02	Octadeca–noids	Jasmonic acids	methyl (+)–7–isojasmonate	methyl (1R,2S)–3–oxo–2–[(2Z)–pent–2–en–1–yl]cyclopentylacetate	225	P
117	FA02	Octadeca–noids	Other Oc-tadecanoids	–	14–oxo–octadecanoic acid	316	P / S
118	FA02	" "	" "	–	3R–hydroxy–octadecanoic acid	318	P
119	FA02	" "	" "	–	8–hydroxy–17–octadecene–9,11–diynoic acid	308	P
120	FA02	" "	" "	–	9–hydroxy–10E,14Z–octadecadien–12–ynoic acid	293	P
121	FA02	" "	" "	–	9R–hydroxy–octadecanoic acid	318	P / S
122	FA02	" "	" "	12–HpOTrE	12–hydroperoxy–9Z,13E,15–octadecatrienoic acid	293 / 311	P / S
123	FA02	" "	" "	18–hydroxy–9R,10S–epoxy–stearicacid	18–hydroxy–9R,10S–epoxy–octadecanoic acid	332 / 337	P / S
124	FA02	" "	" "	18–hydroxy–9S,10R–dihydroxy–stearic acid	18–hydroxy–9S,10R–dihydroxy–octadecanoic acid	350	P / S
125	FA02	" "	" "	2R–hydroxy–oleic acid	2R–hydroxy–9Z–octadecenoic acid	331	P
126	FA02	" "	" "	5,8,12–TriHOME(9)	5,8,12–trihydroxy–9–octadecenoic acid	313	S
127	FA02	" "	" "	5S,8R–DiHODE	5S,8R–dihydroxy–9Z,12Z–octadecadienoic acid	313	S
128	FA02	" "	" "	6,7–dihydroxy stearic acid	6,7–dihydroxy–octadecanoic acid	334	P
129	FA02	" "	" "	7,8–dihydroxy stearic acid	7,8–dihydroxy–octadecanoic acid	334	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
130	FA02	"	"	7S,8S–DiHOTrE	7S,8S–dihydroxy–9Z,12Z,15Z–octadecatrienoic acid	328	S
131	FA02	"	"	9,10,18–TriHOME(12)	9,10,18–trihydroxy–12–octadecenoic acid	331	P / S
132	FA02	"	"	9,12,13,TriHODE	9,12,13–trihydroxy–10,15–octadecadienoic acid	346 / 351	P / S
133	FA02	"	"	9–HODE	9–hydroxy–10E,12Z–octadecadienoic acid	314 / 279	P / S
134	FA02	"	"	9R,10S–dihydroxy–stearic acid	9R,10S–dihydroxy–octadecanoic acid	334	P / S
135	FA02	"	"	9S–HODE–d4	9S–hydroxy–10E,12Z–octadecadienoic acid–d4	301	P / S
136	FA02	"	"	Avenoleic acid	15R–hydroxy–9Z,12Z–octadecadienoic acid	314	P
137	FA02	"	"	α–9,10–DiHODE	(+/–)–9,10–dihydroxy–12Z,15Z–octadecadienoic acid	295	P
138	FA02	"	"	γ– 6(7)–EpODE	6(7)–epoxy–9Z,12Z–octadecadienoic acid	295	S
139	FA03	Eicosanoids	Other Eicosanoids	Dichotellate A	Methyl 12R–(3–methylbutanoyloxy)–5Z,8E,10E,14Z–eicosatetraenoate	436	P / S
140	FA03	Eicosanoids	Prostaglandins	15–deoxy–δ–12,14–PGJ2–d4	11–oxo–5Z,9,12E,14Z–prostatetraenoic acid–d4	303 / 353	P / S
141	FA03	"	"	15–methyl–15R–PGD2	9S,15R–dihydroxy–11–oxo–15–methyl–5Z,13E–prostadienoic acid	367	S
142	FA03	"	"	2,3–dinor–PGE1	9–oxo–11R,15S–dihydroxy–2,3–dinor–13E–prostenoic acid	344	P
143	FA03	"	"	PGD2–d4	9S,15S–dihydroxy–11–oxo–5Z,13E–prostadienoic acid (3,3,4,4–d4)	379	S
144	FA03	"	"	PGF2α isopropyl ester	isopropyl 9S,11R,15S–trihydroxy–5Z,13E–prostadienoate	414	S
145	FA03	"	"	PGF2α methyl ester	methyl 9S,11R,15S–trihydroxy–5Z,13E–prostadienoate	386	P / S
146	FA03	"	"	PGH2–EA	N–(9S,11R–epidioxy–15S–hydroxy–5Z,13E–prostadienoyl)–ethanolamine	394	S
147	FA03	"	"	PGI2–EA	N–(6,9S–epoxy–11R,15S–dihydoxy–5Z,13E–prostadienoyl)–ethanolamine	396	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
148	FA03	" "	" "	Tetranor–PGEM–d6	11R–hydroxy–9,15–dioxo–2,3,4,5–tetranor–prostan–1,20–dioic acid–d6	725	P
149	FA03	Eicosanoids	Leukotrienes	12–keto–10,11,14,15–tetrahydro–LTB4	5S–hydroxy–12–keto–6Z,8E–eicosadienoic acid	339	S
150	FA03	Eicosanoids	Thrombo–xanes	2,3–Dinor–TXB1	9S,11,15S–trihydroxy–2,3–dinor–thrombox–13E–en–1–oic acid	360	P / S
151	FA03	Eicosanoids	Hydroxy/ hydrope–roxyeico–satetra–enoic acids	8R–HETE	8R–hydroxy–5Z,9E,11Z,14Z–eicosatetraenoic acid	303	S
152	FA03	" "	" "	10,11–dihydro–12–oxo–Resolvin E1	12–oxo–5S,18S–dihydroxy–6Z,8E,14Z,16E–eicosatetraenoic acid	368	S
153	FA03	Eicosanoids	Hydroxy/ hydrope–roxyeic–osapenta–enoicacids	12–oxo–Resolvin E1	12–oxo–5S,18S–dihydroxy–6Z,8E,10E,14Z,16E–eicosapentaenoic acid	366	P
154	FA03	Eicosanoids	Hepoxilins	14,15–HxA3–D(11S)	11S,15S–dihydroxy–14R–(S–cysteinyl–glycyl)–5Z,8Z,12E–eicosatrienoic acid	497	S
155	FA03	Eicosanoids	Isoprostanes	7–F2–dihomo–IsoP	1a,1b–dihomo–5,9,11–trihydroxy–6E,14Z–prostadienoic acid–cyclo[8,12]	400	P
156	FA03	" "	" "	8–epi–15–D2c–IsoP	11–oxo–9S,15S–dihydroxy–5Z,13E–prostadienoic acid–cyclo[8S,12S]	335	S
157	FA03	" "	" "	ent–5–epi–5–J2–IsoP	5S–hydroxy–11–oxo–6E,14Z,12–prostatrienoic acid–cyclo[8S,12R]	352	S
158	FA04	Docosanoids	–	4S–hydroperoxy–17S–HDHA	4S–hydroperoxy–17S–hydroxy–5E,7Z,10Z,13Z,15E,19Z–docosahexaenoic acid	394	S
159	FA04	" "	–	7S,8S–epoxy–17R–HDHA	7S,8S–epoxy–17R–hydroxy–4Z,9E,11E,13Z,15E,19Z–docosahexaenoic acid	376	P / S
160	FA04	" "		Adrenic acid	7,10,13,16–docosatetraenoic acid oder 7Z,10Z,13Z,16Z–docosatetraenoic acid	350	P / S
161	FA04	" "	–	DPA	7Z,10Z,13Z,16Z,19Z–docosapentaenoic acid	353	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr. Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
*162*FA05	Fatty alcohols	–	–	1,3–Hexadecadien–1–ol	239	S
*163*FA05	" "	–	–	10,11–Difluoro–8E,10E–dodecadien–1–ol	201	P / S
*164*FA05	" "	–	–	11Z–heptadecen–1–ol	255 / 272	P / S
*165*FA05	" "	–	–	2,2,9,9–tetramethyl–undecan–1,10–diol	248	P
*166*FA05	" "	–	–	2Z,13Z–Octadecadien–1–ol	267	S
*167*FA05	" "	–	–	6Z,11Z–hexadecadien–1–ol	239	P
*168*FA05	" "	–	–	8Z,10E–Dodecadien–1–ol	183	P
*169*FA05	" "	–	–	9,12,15–Octadecatrien–1–ol	282	S
*170*FA05	" "	–	anteiso–1,2–octadecanediol	3–methylheptadecane–1,2–diol	309 / 304	P / S
*171*FA05	" "	–	Falcarindiol	1,9–heptadecadien–4,6–diyn–3,8–diol oder Heptadeca–1,9E–dien–4,6–diyne–3R,8S– diol oder (3R,8S,9Z)–heptadeca–1,9–dien–4,6– diyne–3,8–diol	243 / 278	P / S
*172*FA05	" "	–	iso–1,2–heptadecanediol	2–methylhexadecane–1,2–diol	290	S
*173*FA05	" "	–	Persin	1–acetoxy–2–hydroxy–12Z,15Z–heneicosadien–4–one	381	S
*174*FA06	Fatty aldehydes	–	–	11Z,13–Tetradecadienal	209	S
*175*FA06	" "	–	–	13Z–Hexadecen–11–ynal	252	S
*176*FA06	" "	–	–	2,4,6,8–decatetraenal	181	P
*177*FA06	" "	–	–	3,7–Dimethyl–8,11–dioxo–2E,6E,9E–dodecatrienal	252 / 217	P / S
*178*FA06	" "	–	–	4,10–undecadiynal	163	P / S
*179*FA06	" "	–	–	8Z,10Z–Tetradecadienal	209	S
*180*FA06	" "	–	–	9Z,12Z,15Z–Octadecatrienal	263	S
*181*FA06	" "	–	2–hydroxyhexadecanal	2–hydroxyhexadecanal	257	P / S
*182*FA06	" "	–	Muconic dialdehyde	2,4–hexadienedial	111	S
*183*FA06	" "			7Z,11Z–Hexadecadienal	237	P
*184*FA07	Fatty esters	Wax mono–esters	–	(E)–3,7–Dimethyl–2,6–octadienyl nonanoate	312	S
*185*FA07	" "	" "	–	(Z)–3–Hexenyl 3–methylbutanoate	167	S
*186*FA07	" "	" "	–	11Z–octadecenyl hexadecanoate	525	S
*187*FA07	" "	" "	–	12–methyl–tridecanyl 10–methyl–hexadecanoate	485	S
*188*FA07	" "	" "	–	14,14,14–Trifluoro–11E–tetradecenyl acetate	326 / 309.	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
189	FA07	"	"	–	16–methyl–heptadecanyl tetradecanoate	499	P / S
190	FA07	"	"	–	1–Methylbutyl 3–methylbutanoate	181	P
191	FA07	"	"	–	3E,13E–Octadecadienyl acetate	326	P
192	FA07	"	"	–	3E,8Z,11Z–Tetradecatrienyl acetate	273	P / S
193	FA07	"	"	–	4E,9Z–Tetradecadienyl acetate	235 / 285	P / S
194	FA07	"	"	–	4E–Tridecenyl acetate	273	P / S
195	FA07	"	"	–	5E–Dodecenyl acetate	244	P
196	FA07	"	"	–	9E–Hexadecenyl acetate	305 / 300	P / S
197	FA07	"	"	–	decyl octanoate	302	P / S
198	FA07	"	"	–	formyl 7–oxo–11E–tetradecenoate	255	P / S
199	FA07	"	"	–	hexadecyl butyrate	295	P
200	FA07	"	"	–	Methyl (E)–2,6,10–trimethyl–5,9–undecadienoate	221	S
201	FA07	"	"	–	octadecyl 11E–hexadecenoate	525	P
202	FA07	"	"	Arachidyl linoleate	eicosanyl 9Z,12Z–octadecadienoate	577	S
203	FA07	"	"	Behenyl linoleate	docosanyl 9Z,12Z–octadecadienoate	607	S
204	FA07	"	"	Behenyl linolenate	docosanyl 9Z,12Z,15Z–octadecatrienoate	605	S
205	FA07	"	"	Behenyl palmitoleate	docosanyl 9Z–hexadecenoate	581	S
206	FA07	"	"	Heptadecyl palmitoleate	Heptadecyl 9Z–hexadecenoate	511	P / S
207	FA07	"	"	Heptadecyl–palmitate	heptadecyl hexadecanoate	513	S
208	FA07	"	"	Lauryl oleate	dodecanyl 9Z–octadecenoate	468	P / S
209	FA07	"	"	Linoleyl arachidate	9Z,12Z–octadecadienyl eicosanoate	579	S
210	FA07	"	"	Linoleyl myristate	9Z,12Z–octadecadienyl tetradecanoate	494	S
211	FA07	"	"	Myristoleyl behenate	9Z–tetradecenyl docosanoate	553	P
212	FA07	"	"	Myristyl laurate	tetradecanyl dodecanoate	414	P / S
213	FA07	"	"	Myristyl oleate	tetradecanyl 9Z–octadecenoate	497	P / S
214	FA07	"	"	Nonadecyl palmitoleate	Nonadecyl 9Z–hexadecenoate	539	S
215	FA07	"	"	Oleyl oleate	9Z–octadecenyl 9Z–octadecenoate	551	P / S
216	FA07	"	"	Palmityl linoleate	hexadecanyl 9Z,12Z–octadecadienoate	523	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
217	FA07	"	"	Palmityl myristate	hexadecanyl tetradecanoate	470	P / S
218	FA07	"	"	Palmityl stearate	hexadecanyl octadecanoate	527	P / S
219	FA07	"	"	Stearyl linolenate	octadecanyl 9Z,12Z,15Z–octadecatrienoate	564	S
220	FA07	Fatty esters	Lactones	–	3–acetyl–3–methyldihydrofuran–2(3H)–one	175 / 125	P / S
221	FA07	"	"	–	Dihydro–3–hydroxy–3–(1–hydroxyethyl)–4–methylfuran–2(3H)–one	143 / 178	P / S
222	FA07	Fatty esters	Fatty acyl carnitines	–	O–butanoyl–carnitine	232	P
223	FA07	"	"	–	O–octanoyl–R–carnitine	310 / 288	P / S
224	FA07	"	"	hexadecanedioic acid mono–L–carnitine ester	–	447	S
225	FA08	Fatty amides	Primary amides	–	dodecanamide	200	P
226	FA08	"	"	–	pentanamide	84	P / S
227	FA08	"	"	decanamide	decanamide	172	S
228	FA08	"	"	Linoleamide	9Z,12Z–octadecadienamide	280	S
229	FA08	"	"	Palmitamide	Hexadecanamide	256 / 273	P / S
230	FA08	"	"	Palmitoleamide	9Z–hexadecenamide	276	P / S
231	FA08	"	"	Stearamide	Octadecanamide	284	P / S
232	FA08	Fatty amides	N–acyl amines	–	N–propyl–16,16–dimethyl–5Z,8Z,11Z,14Z–docosatetraenoyl amine	402	S
233	FA08	"	"	N–(2–phenoxy–ethyl) arachidonoyl amine	N–(2–phenoxy–ethyl)–5Z,8Z,11Z,14Z–eicosatetraenoyl amine	446	S
234	FA08	"	"	N–palmitoyl glycine	N–hexadecanoyl–glycine	296 / 314	P / S
235	FA08	"	"	N–palmitoyl phenylalanine	N–hexadecanoyl–phenylalanine	436	P
236	FA08	"	"	N–stearoyl serine	N–octadecanoyl–serine	404	P
237	FA08	"	"	N–stearoyl alanine	N–octadecanoyl–alanine	356	S
238	FA08	Fatty amides	Fatty acyl homoserine lactones	–	N–heptanoyl–homoserine lactone	246	S
239	FA08	Fatty amides	N–acyl ethanol–amines (endocannabinoids)	Anandamide (18:2, n–6)	N–(9Z,12Z–octadecadienoyl)–ethanolamine	324	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
240	FA08	" "	" "	Anandamide (20:2, n–6)	N–(11Z,14Z–eicosadienoyl)–ethanolamine	352	S
241	FA08	" "	" "	Arachidonoyl–EA(d8)	N–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–ethanolamine(d8)	394	P
242	FA08	" "	" "	Dihomo–γ–linolenoyl–EA	N–(8Z,11Z,14Z–eicosatrienoyl)–ethanolamine	367	P
243	FA08	" "	" "	Lauroyl–EA	N–(Dodecanoyl)–ethanolamine	244	P
244	FA11	Hydrocarbons		–	1–Octacosene	415	S
245	FA11	" "	–	–	4,6–Dimethyl–2E,4E,6E–nonatriene	151	P
246	FA11	" "	–	–	6,10–Dimethyl–5(E),9–undecadien–2–one	227 / 195	P / S
247	FA11	" "	–	–	7–Heptacosene	401	S
248	FA12	Oxygenated hydrocarbons	–	–	(3S,4R)–(6S,7R)–Diepoxy–9Z–heneicosene	340	P
249	FA12	" "	–	–	10–Heptadecen–2–one	235 / 270	P / S
250	FA12	" "	–	–	2–methyl–7R,8S–Epoxy–octadecane	300	P / S
251	FA12	" "	–	–	2S–Hydroxyhexan–3–one	99	S
252	FA12	" "	–	–	3E–Nonen–2–one	141	S
253	FA12	" "	–	–	5–Ethylundecane–2,4–dione	195	P / S
254	FA12	" "	–	–	6S,7R–Epoxy–3Z,9Z–octadecadiene	282 / 265	P / S
255	FA12	" "	–	–	7–Ethyl–4E–undecen–6–one	219	S
256	FA12	" "	–	hentriacontane–14,16–dione	hentriacontane–14,16–dione	482	P / S
257	FA13	Fatty acyl glycosides	Fatty acyl glycosides of mono– and disaccharides	–	1–O–α–D–glucopyranosyl–1,2–eicosandiol	494	S
	Glycerophospholipids (GPs)						
1	GP00	Other Glycerophospholipids	–	–	1,2–diacyl–3–O–(phospho–2'–O–(1'–amino)–2',3',4',5'–pentanetetrol)–sn–glycerol	390	P
2	GP01	Glycero–phospho–cholines (PCs)	Diacylgly–cerophos–phocholines	PC(14:0/21:0)	1–tetradecanoyl–2–heneicosanoyl–glycero–3–phosphocholine	809	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
3	GP01	" "	" "	PC(14:1(9Z)/18:1 (9Z))	1–(9Z–tetradecenoyl)–2–(9Z–octadecenoyl)–glycero–3–phosphocholine	753	S
4	GP01	" "	" "	PC(18:0/18:0)	1,2–dioctadecanoyl–sn–glycero–3–phosphocholine	823	P / S
5	GP01	" "	" "	PC(18:0/20:5(9Z,11Z, 13Z,15Z,17Z))	1–octadecanoyl–2–(9Z,11Z,13Z,15Z,17Z–eicosapentaenoyl)–sn–glycero–3–phosphocholine	791	S
6	GP01	" "	" "	PC(18:3(9Z,12Z,15Z)/ 17:0)	1–(9Z,12Z,15Z–octadecatrienoyl)–2–heptadecanoyl–glycero–3–phosphocholine	771	S
7	GP01	" "	" "	PC(18:4(6Z,9Z,12Z, 15Z)/18:0)	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–octadecanoyl–glycero–3–phosphocholine	805	S
8	GP01	" "	" "	PC(20:2(11Z,14Z)/ 17:0)	1–(11Z,14Z–eicosadienoyl)–2–heptadecanoyl–glycero–3–phosphocholine	783	S
9	GP01	" "	" "	PC(20:3(8Z,11Z,14Z)/ 12:0)	1–(8Z,11Z,14Z–eicosatrienoyl)–2–dodecanoyl–glycero–3–phosphocholine	729	P / S
10	GP01	" "	" "	PC(20:4(5Z,8Z,11Z, 14Z)/17:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–2–heptadecanoyl–glycero–3–phosphocholine	797	P
11	GP01	" "	" "	PC(20:5(5Z,8Z,11Z, 14Z,17Z)/14:0)	1–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–2–tetradecanoyl–glycero–3–phosphocholine	785	S
12	GP01	" "	" "	PC(20:5(5Z,8Z,11Z, 14Z,17Z)/15:0)	1–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–2–pentadecanoyl–glycero–3–phosphocholine	749	P
13	GP01	" "	" "	PC(20:5(5Z,8Z,11Z, 14Z,17Z)/17:0)	1–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–2–heptadecanoyl–glycero–3–phosphocholine	777	S
14	GP01	" "	" "	PC(22:6(4E,7E,10E, 13E,16E,19E)/16:0)	1–(4E,7E,10E,13E,16E,19E–docosahexaenoyl)–2–hexadecanoyl–sn–glycero–3–phosphocholine	829	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
15	GP01	" "	" "	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/16:1(9Z))	1–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–2–(9Z–hexadecenoyl)–glycero–3–phosphocholine	805	P / S
16	GP01	Glycero–phospho–cholines (PCs)	1–(1Z–alkenyl),2–acylglycero–phospho-cholines	PC(O–16:0/1:0)	1–hexadecyl–2–formyl–sn–glycero–3–phosphocholine	510 / 532	P / S
17	GP01	" "	" "	PC(O–16:0/19:0)	1–hexadecyl–2–nonadecanoyl–sn–glycero–3–phosphocholine	763	P
18	GP01	" "	" "	PC(O–16:0/22:5(7Z,10Z,13Z,16Z,19Z))	1–hexadecyl–2–(7Z,10Z,13Z,16Z,19Z–docosapentaenoyl)–sn–glycero–3–phosphocholine	795	P / S
19	GP01	" "	" "	PC(O–18:0/1:0)	1–octadecyl–2–formyl–sn–glycero–3–phosphocholine	538	S
20	GP01	" "	" "	PC(O–18:0/19:0)	1–octadecyl–2–nonadecanoyl–glycero–3–phosphocholine	823	P
21	GP01	" "	" "	PC(P–14:0/18:1(9Z))	1–O–(1Z–tetradecenyl)–2–(9Z–octadecenoyl)–sn–glycero–3–disaccharides [FA1301]	749 / 755	P / S
22	GP01	" "	" "	PC(P–16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	1–(1Z–hexadecenyl)–2–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–sn–glycero–3–phosphocholine	791	P
23	GP01	" "	" "	PC(P–18:0/2:0)	1–(1Z–octadecenyl)–2–acetyl–sn–glycero–3–phosphocholine	550	P
24	GP01	" "	" "	PC(P–20:0/20:4(5Z,8Z,11Z,14Z))	1–(1Z–eicosenyl)–2–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–glycero–3–phosphocholine	845	P
25	GP01	Glycero–phospho–cholines (PCs)	Monoacyl–glycero–phospho-cholines	PC(0:0/16:0)	2–hexadecanoyl–sn–glycero–3–phosphocholine	496	P / S
26	GP01	" "	" "	PC(0:0/20:4(5Z,8Z,11Z,14Z))	2–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–sn–glycero–3–phosphocholine	544	P / S
27	GP01	" "	" "	PC(13:0/0:0)	1–tridecanoyl–sn–glycero–3–phosphocholine	454	P
28	GP01	" "	" "	PC(15:0/0:0)	1–pentadecanoyl–sn–glycero–3–phosphocholine	482	P
29	GP01	" "	" "	PC(15:1(9Z)/0:0)	1–(9Z–pentadecenoyl)–glycero–3–phosphocholine	480	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
30	GP01	" "	" "	PC(16:1(9Z)/0:0)	1–(9Z–hexadecenoyl)–sn–glycero–3–phosphocholine	494	S
31	GP01	" "	" "	PC(18:0/0:0)	1–octadecanoyl–sn–glycero–3–phosphocholine	546 / 524	P / S
32	GP01	" "	" "	PC(18:1(9Z)/0:0)[rac]	1–(9Z–octadecenoyl)–sn–glycero–3–phosphocholine	522	P
33	GP01	" "	" "	PC(18:2(9Z,12Z)/0:0)	1–(9Z,12Z–octadecadienoyl)–sn–glycero–3–phosphocholine	520	P / S
34	GP01	" "	" "	PC(18:4(6Z,9Z,12Z, 15Z)/0:0)	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–glycero–3–phosphocholine	548	S
35	GP01	" "	" "	PC(20:0/0:0)	1–eicosanoyl–sn–glycero–3–phosphocholine	552	S
36	GP01	" "	" "	PC(20:3(8Z,11Z,14Z) /0:0)	1–(8Z,11Z,14Z–eicosatrienoyl)–glycero–3–phosphocholine	578	P / S
37	GP01	" "	" "	PC(20:5(5Z,8Z,11Z, 14Z,17Z)/0:0)	1–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–sn–glycero–3–phosphocholine	542	P / S
38	GP01	" "	" "	PC(22:1(11Z)/0:0)	1–(11Z–docosenoyl)–glycero–3–phosphocholine	578	S
39	GP01	" "	" "	PC(22:4(7Z,10Z,13Z, 16Z)/0:0)	1–(7Z,10Z,13Z,16Z–docosatetraenoyl)–glycero–3–phosphocholine	572	P / S
40	GP01	" "	" "	PC(22:6(4Z,7Z,10Z, 13Z,16Z,19Z)/0:0)	1–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–sn–glycero–3–phosphocholine	568	P
41	GP01	" "	" "	PC(O–16:0/0:0)	1–hexadecyl–sn–glycero–3–phosphocholine	482	P / S
42	GP01	" "	" "	PC(O–16:1(11Z)/0:0)	1–(11Z–hexadecenyl)–sn–glycero–3–phosphocholine	480	P
43	GP02	Glycero–phospho–ethanol–amines (PEs)	Diacylgly–cerophospho–ethanolamines	PE(15:0/12:0)	1–pentadecanoyl–2–dodecanoyl–glycero–3–phosphoethanolamine	604	P / S
44	GP02	" "	" "	PE(15:1(9Z)/21:0)	1–(9Z–pentadecenoyl)–2–heneicosanoyl–glycero–3–phosphoethanolamine	729	P / S
45	GP02	" "	" "	PE(16:1(9Z)/22:0)	1–(9Z–hexadecenoyl)–2–docosanoyl–glycero–3–phosphoethanolamine	775	S
46	GP02	" "	" "	PE(18:0(10(R)Me)/ 16:0)	1–((R)–10–methyloctadecanoyl)–2–hexadecanoyl–sn–glycero–3–phosphoethanolamine	735	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
47	GP02	" "	" "	PE(18:0/15:0)	1–octadecanoyl–2–pentadecanoyl–glycero–3–phosphoethanolamine	729	P
48	GP02	" "	" "	PE(18:1(9Z)/21:0)	1–(9Z–octadecenoyl)–2–heneicosanoyl–glycero–3–phosphoethanolamine	821	P / S
49	GP02	" "	" "	PE(18:2(9Z,12Z)/18:0)	1–(9Z,12Z–octadecadienoyl)–2–octadecanoyl–glycero–3–phosphoethanolamine	745	S
50	GP02	" "	" "	PE(18:2(9Z,12Z)/21:0)	1–(9Z,12Z–octadecadienoyl)–2–heneicosanoyl–glycero–3–phosphoethanolamine	769	P / S
51	GP02	" "	" "	PE(18:3(9Z,12Z,15Z)/19:0)	1–(9Z,12Z,15Z–octadecatrienoyl)–2–nonadecanoyl–glycero–3–phosphoethanolamine	739	P
52	GP02	" "	" "	PE(18:4(6Z,9Z,12Z,15Z)/22:0)	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–docosanoyl–glycero–3–phosphoethanolamine	779	P
53	GP02	" "	" "	PE(20:3(8Z,11Z,14Z)/18:0)	1–(8Z,11Z,14Z–eicosatrienoyl)–2–octadecanoyl–glycero–3–phosphoethanolamine	771	P
54	GP02	" "	" "	PE(20:4(5Z,8Z,11Z,14Z)/20:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–2–eicosanoyl–glycero–3–phosphoethanolamine	797	S
55	GP02	" "	" "	PE(20:5(5Z,8Z,11Z,14Z,17Z)/19:1(9Z))	1–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–2–(9Z–nonadecenoyl)–glycero–3–phosphoethanolamine	779	S
56	GP02	" "	" "	PE(20:5(5Z,8Z,11Z,14Z,17Z)/20:0)	1–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–2–eicosanoyl–glycero–3–phosphoethanolamine	777	P
57	GP02	" "	" "	PE(21:0/16:0)	1–heneicosanoyl–2–hexadecanoyl–glycero–3–phosphoethanolamine	801	S
58	GP02	" "	" "	PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:3(6Z,9Z,12Z))	1–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–2–(6Z,9Z,12Z–octadecatrienoyl)–glycero–3–phosphoethanolamine	804	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
59	GP02	" "	" "	PE(22:6(4Z,7Z,10Z, 13Z,16Z,19Z)/21:0)	1–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–2–heneicosanoyl–glycero–3–phosphoethanolamine	835	P
60	GP02	Glycero–phospho–ethanol–amines (PEs)	1–alkyl,2–acylglycero–phosphoetha–nolamines	PE(O–16:0/13:0)	1–hexadecyl–2–tridecanoyl–glycero–3–phosphoethanolamine	654	S
61	GP02	Glycero–phospho–ethanol–amines (PEs)	1–(1Z–alkenyl),2–acylglycero–phosphoetha–nolamines	PE(P–16:0/22:4(7Z,10Z,13Z, 16Z))	1–(1Z–hexadecenyl)–2–(7Z,10Z,13Z,16Z–docosatetraenoyl)–glycero–3–phosphoethanolamine	753	P / S
62	GP02	" "	" "	PE(P–20:0/22:1(11Z))	1–(1Z–eicosenyl)–2–(11Z–docosenoyl)–glycero–3–phosphoethanolamine	832	P
63	GP02	Glycero–phospho–ethanol–amines (PEs)	Monoacyl–glycerophos–phoethanol–amines	PE(16:0/0:0)	1–hexadecanoyl–sn–glycero–3–phosphoethanolamine	454	S
64	GP02	" "	" "	PE(17:0/0:0)	1–heptadecanoyl–glycero–3–phosphoethanolamine	468	P / S
65	GP02	" "	" "	PE(18:0/0:0)	1–octadecanoyl–sn–glycero–3–phosphoethanolamine	482	S
66	GP02	" "	" "	PE(18:1(9Z)/0:0)	1–(9Z–octadecenoyl)–sn–glycero–3–phosphoethanolamine	480	S
67	GP02	" "	" "	PE(20:4(5Z,8Z,11Z, 14Z)/0:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–sn–glycero–3–phosphoethanolamine	502	P
68	GP02	" "	" "	PE(22:0/0:0)	1–docosanoyl–glycero–3–phosphoethanolamine	538	P
69	GP02	Glycero–phospho–ethanol–amines (PEs)	Monoalkyl–glycerophos–phoetha–nolamines	PE(O–18:1(9Z)/0:0)	1–(9Z–octadecenyl)–sn–glycero–3–phosphoethanolamine	466	S
70	GP02	Glycero–phospho–ethanol–amines (PEs)	1Z–alkenylgly–cerophos–phoetha–nolamines	PE(P–18:0/0:0)	1–(1Z–octadecenyl)–sn–glycero–3–phosphoethanolamine	466	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
71	GP03	Glycero–phospho-serines (PSs)	Diacylgly–cerophos–phoserines	PS(17:2(9Z,12Z)/21:0)	1–(9Z,12Z–heptadecadienoyl)–2–heneicosanoyl–glycero–3–phosphoserine	799	S
72	GP03	" "	" "	PS(18:2(9Z,12Z)/13:0)	1–(9Z,12Z–octadecadienoyl)–2–tridecanoyl–glycero–3–phosphoserine	718	S
73	GP03	" "	" "	PS(18:3(6Z,9Z,12Z)/22:0)	1–(6Z,9Z,12Z–octadecatrienoyl)–2–docosanoyl–glycero–3–phosphoserine	825	P
74	GP03	" "	" "	PS(20:1(11Z)/16:0)	1–(11Z–eicosenoyl)–2–hexadecanoyl–glycero–3–phosphoserine	791	S
75	GP03	" "	" "	PS(20:3(8Z,11Z,14Z)/20:0)	1–(8Z,11Z,14Z–eicosatrienoyl)–2–eicosanoyl–glycero–3–phosphoserine	825	S
76	GP03	Glycero–phospho-serines (PSs)	1–alkyl,2–acylglycero–phospho-serines	PS(O–18:0/15:0)	1–octadecyl–2–pentadecanoyl–glycero–3–phosphoserine	737	P
77	GP03	" "	" "	PS(O–20:0/19:0)	1–eicosyl–2–nonadecanoyl–glycero–3–phosphoserine	803	P / S
78	GP03	" "	" "	PS(O–20:0/22:1(11Z))	1–eicosyl–2–(11Z–docosenoyl)–glycero–3–phosphoserine	843	S
79	GP03	Glycero–phospho-serines (PSs)	1–(1Z–alkenyl),2–acylglycero–phospho-serines	PS(P–16:0/13:0)	1–(1Z–hexadecenyl)–2–tridecanoyl–glycero–3–phosphoserine	678	S
80	GP03	" "	" "	PS(P–16:0/18:0)	1–(1Z–hexadecenyl)–2–octadecanoyl–glycero–3–phosphoserine	781 / 749	P / S
81	GP03	" "	" "	PS(P–16:0/19:1(9Z))	1–(1Z–hexadecenyl)–2–(9Z–nonadecenoyl)–glycero–3–phosphoserine	761	S
82	GP03	" "	" "	PS(P–16:0/20:0)	1–(1Z–hexadecenyl)–2–eicosanoyl–glycero–3–phosphoserine	759	S
83	GP03	" "	" "	PS(P–16:0/20:1(11Z))	1–(1Z–hexadecenyl)–2–(11Z–eicosenoyl)–glycero–3–phosphoserine	813	P
84	GP03	" "	" "	PS(P–16:0/22:1(11Z))	1–(1Z–hexadecenyl)–2–(11Z–docosenoyl)–glycero–3–phosphoserine	785	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
85	GP03	" "	" "	PS(P–18:0/14:1(9Z))	1–(1Z–octadecenyl)–2–(9Z–tetradecenoyl)–glycero–3–phosphoserine	751	P
86	GP03	" "	" "	PS(P–18:0/20:0)	1–(1Z–octadecenyl)–2–eicosanoyl–glycero–3–phosphoserine	827	P / S
87	GP03	" "	" "	PS(P–20:0/19:1(9Z))	1–(1Z–eicosenyl)–2–(9Z–nonadecenoyl)–glycero–3–phosphoserine	799	P
88	GP03	" "	" "	PS(P–20:0/22:0)	1–(1Z–eicosenyl)–2–docosanoyl–glycero–3–phosphoserine	843	P
89	GP03	Glycero–phospho-serines (PSs)	Monoacyl–glycerophos–phoserines	PS(13:0/0:0)	1–tridecanoyl–sn–glycero–3–phosphoserine	473	S
90	GP03	" "	" "	PS(O–18:0/0:0)	1–octadecyl–glycero–3–phosphoserine	494	P
91	GP03	" "	" "	PS(O–20:0/0:0)	1–eicosyl–glycero–3–phosphoserine	522	S
92	GP04	Glycero–phospho–glycerols (PGs)	Diacyl–glycerophos–phoglyc-erols	PG(18:4(6Z,9Z,12Z,15Z)/12:0)	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–dodecanoyl–glycero–3–phospho–(1'–sn–glycerol)	704	S
93	GP04	Glycero–phospho–glycerols (PGs)	Monoacyl–glycerophos–phoglyc-erols	PG(15:0/0:0)	1–pentadecanoyl–glycero–3–phospho–(1'–sn–glycerol)	488	P
94	GP06	Glycero–phospho–inositols (PIs)	Diacyl–glycerophos–phoinosi-tols	PI(12:0/22:1(11Z))	1–dodecanoyl–2–(11Z–docosenoyl)–glycero–3–phospho–(1'–myo–inositol)	855	S
95	GP06	" "	" "	PI(14:1(9Z)/22:1 (11Z))	1–(9Z–tetradecenoyl)–2–(11Z–docosenoyl)–glycero–3–phospho–(1'–myo–inositol)	881	P / S
96	GP06	" "	" "	PI(17:2(9Z,12Z)/17:0)	1–(9Z,12Z–heptadecadienoyl)–2–heptadecanoyl–glycero–3–phospho–(1'–myo–inositol)	853	P / S
97	GP06	" "	" "	PI(18:3(6Z,9Z,12Z)/ 20:2(11Z,14Z))	1–(6Z,9Z,12Z–octadecatrienoyl)–2–(11Z,14Z–eicosadienoyl)–glycero–3–phospho–(1'–myo–inositol)	903	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
98	GP06	" "	" "	PI(18:4(6Z,9Z,12Z, 15Z)/20:0)	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–eicosanoyl–glycero–3–phospho–(1'–myo–inositol)	905	P / S
99	GP06	" "	" "	PI(19:1(9Z)/15:0)	1–(9Z–nonadecenoyl)–2–pentadecanoyl–glycero–3–phospho–(1'–myo–inositol)	855	P
100	GP06	" "	" "	PI(20:4(5Z,8Z,11Z ,14Z)/16:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–2–hexadecanoyl–glycero–3–phospho–(1'–myo–inositol)	877	P / S
101	GP06	Glycero–phospho–inositols (PIs)	1–alkyl,2–acylgly–cerophos-phoinosi-tols	PI(O–16:0/16:0)	1–hexadecyl–2–hexadecanoyl–glycero–3–phospho–(1'–myo–inositol)	815	S
102	GP06	Glycero–phospho–inositols (PIs)	Monoacyl–glycerophos–phoinosi-tols	PI(18:0/0:0)	1–octadecanoyl–sn–glycero–3–phospho–(1'–myo–inositol)	601	P / S
103	GP06	" "	" "	PI(20:4(5Z,8Z,11Z, 14Z)/0:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–sn–glycero–3–phospho–(1'–myo–inositol)	638	P
104	GP10	Glycero–phosphates (PAs)	Diacylgly–cerophos–phates	PA(16:1(9Z)/16:0)	1–(9Z–hexadecenoyl)–2–hexadecanoyl–glycero–3–phosphate	647	P / S
105	GP10	" "	" "	PA(18:4(6Z,9Z,12Z, 15Z)/17:2(9Z,12Z))	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–(9Z,12Z–heptadecadienoyl)–glycero–3–phosphate	679	P / S
106	GP10	" "	" "	PA(22:6(4Z,7Z,10Z, 13Z,16Z,19Z)/17:0)	1–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–2–heptadecanoyl–glycero–3–phosphate	717	P
107	GP10	Glycero–phosphates (PAs)	1–alkyl,2–acylglycero–phosphates	PA(O–20:0/17:0)	1–eicosyl–2–heptadecanoyl–glycero–3–phosphate	738	P
108	GP10	" "	" "	PA(P–20:0/0:0)	1–(1Z–eicosenyl)–glycero–3–phosphate	451	P / S
109	GP10	Glycero–phosphates (PAs)	Monoacyl–glycero–phosphates	PA(0:0/18:1(9Z))	2–(9Z–octadecenoyl)–sn–glycero–3–phosphate	454	P / S
110	GP10	" "	" "	PA(16:0/0:0)	1–hexadecanoyl–sn–glycero–3–phosphate	428	P / S
111	GP10	" "	" "	PA(18:0/0:0)	1–octadecanoyl–sn–glycero–3–phosphate	456	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
112	GP10	" "	" "	PA(18:2(9Z,12Z)/0:0)	1–(9Z,12Z–octadecadienoyl)–glycero–3–phosphate	452	P / S
113	GP10	" "	" "	PA(20:4(5Z,8Z,11Z,14Z)/0:0)	1–(5Z,8Z,11Z,14Z–eicosatetraenoyl)–sn–glycero–3–phosphate	476	P / S
114	GP20	Oxidized glycero–phospho-lipids	Oxidized glycero–phospho-cholines	PC(16:0/9:0(CHO))	1–hexadecanoyl–2–(9–oxo–nonanoyl)–sn–glycero–3–phosphocholine	650	P
115	GP20	Oxidized glycero–phospho-lipids	Oxidized glycero–phospho–ethanol–amines	PE(P–16:0/20:4(5Z,8Z,10E,14Z)(12OH[S]))	1–O–(1Z–hexadecenyl)–2–(12S–hydroxy–5Z,8Z,10E,14Z–eicosatetraenoyl)–sn–glycero–3–phosphoethanolamine	741	P / S
116	GP20	" "	" "	PE(P–18:0/20:4(5Z,8Z,10E,14Z)(12OH[S]))	1–O–(1Z–octadecenyl)–2–(12S–hydroxy–5Z,8Z,10E,14Z–eicosatetraenoyl)–sn–glycero–3–phosphoethanolamine	769	P / S
	Sterol Lipids (STs)						
1	ST01	Sterols	Cholesterol and derivatives	–	(25S)–5α–cholestan–3β,6α,7β,8β,15α,16β,26–heptol	502	P / S
2	ST01	" "	" "	15α–hydroxycholestene	5α–cholest–8(14)–en–3β,15α–diol	385	P / S
3	ST01	" "	" "	4β–(hydroxymethyl)–4α–methyl–5α–cholest–7–en–3β–ol	4β–(hydroxymethyl)–4–methyl–5α–cholest–7–en–3β–ol 4β–(hydroxymethyl)–4α–methyl–5α–cholest–7–en–3β–ol	448	P / S
4	ST01	" "	" "	Cholestan eskeleton	–	373	S
5	ST01	" "	" "	Cucurbitacin E	–	574	P / S
6	ST01	" "	" "	Cucurbitacin O	–	536	P
7	ST01	" "	" "	DHCEO	3β,5α–dihydroxycholest–7–en–6–one	417	P
8	ST01	Sterols	Steryl esters	14:1 Cholesteryl ester	cholest–5–en–3b–yl (9Z–tetradecenoate)	618	P
9	ST01	" "	" "	18:1 Cholesteryl ester	cholest–5–en–3b–yl (9Z–octadecenoate)	669	P / S
10	ST01	" "	" "	18:2 Cholesteryl ester	cholest–5–en–3β–yl (9Z,12Z–octadecadienoate)	667	S
11	ST01	" "	" "	18:3 Cholesteryl ester	cholest–5–en–3β–yl (9Z,12Z,15Z–octadecatrienoate)	665	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
12	ST01	" "	" "	20:4 Cholesteryl ester	cholest–5–en–3β–yl (5Z,8Z,11Z,14Z–eicosatetraenoate)	691	P / S
13	ST01	" "	" "	20:5 Cholesteryl ester	cholest–5–en–3β–yl (5Z,8Z,11Z,14Z,17Z–eicosapentaenoate)	689	P / S
14	ST01	" "	" "	22:6 Cholesteryl ester	cholest–5–en–3β–yl (4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoate)	715	P
15	ST01	" "	" "	zymosteryl oleate	5α–cholesta–8,24–dien–3β–yl (9Z)–octadec–9–enoate	667	P
16	ST01	Sterols	Ergosterols and C24–methyl derivatives	–	4α,14α–dimethyl–24–methylene–cholest–7,9(11)–dien–3β–ol	442	S
17	ST01	" "	" "	Nebrosteroid L	3β–hydroxy–6β–acetoxy–24–methylene–cholest–5–en–19–al	488	S
18	ST01	" "	" "	Nebrosteroid M	4α–methyl–24–methylene–cholestan–3β,8β,11β–triol	464	S
19	ST01	" "	" "	Xestosterol	24–methylene,26,27–dimethylcholest–5–en–3β–ol	444 / 427	P / S
20	ST01	Sterols	Stigmasterols and C24–ethyl derivatives	–	24–ethyl–5α–cholest–25–en–3α,12α,16α–triol	464 / 429	P / S
21	ST01	Sterols	Cycloartanols and derivatives	cimicifoetiside A	15α,25–dihydroxy–16β,23R:16α,24S–diepoxy–9β,19–cyclolanostan–3β–yl 2–O–acetyl–α–L–arabinopyranoside	680	P / S
22	ST01	Sterols	Cardanolides and derivatives	Digitoxigenin	3–β,14β–dihydroxy–5–β–card–20(22)–enolide	375	P
23	ST01	" "	" "	gitoxigenin	3β,14,16β–trihydroxy–5β–card–20(22)–enolide	391	P / S
24	ST02	Steroids	C19 steroids (androgens) and derivatives	5α–androstane–3β,17α–diol	5α–androstane–3β,17α–diol	310 / 325	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
25	ST02	Steroids	C21 steroids (gluco/mineralocorticoids, progestogens) and derivatives	(20R)–20–hydroxypregn–4–en–3–one	20R–hydroxypregn–4–en–3–one	334	P / S
26	ST02	" "	" "	17,20–dihydroxypregn–4–en–3–one	17,20–dihydroxypregn–4–en–3–one	350	S
27	ST02	" "	" "	3–hydroxy–5β–pregnan–20–one	3–hydroxy–5β–pregnan–20–one	336	P / S
28	ST02	" "	" "	megestrol	17–hydroxypregna–4,6–diene–3,20–dione	329	S
29	ST03	Secosteroids	Vitamin D3 and derivatives	(24S)–1α,24–dihydroxy–22–oxavitamin D3 / (24S)–1α,24–dihydroxy–22–oxacholecalciferol	(5Z,7E)–(1S,3R,24S)–22–oxa–9,10–seco–5,7,10(19)–cholestatriene–1,3,24–triol	441	S
30	ST03	" "	" "	(25R)–1α,25,26–trihydroxy–22–oxavitamin D3 / (25R)–1α,25,26–trihydroxy–22–oxacholecalciferol	(5Z,7E)–(1S,3R,25R)–22–oxa–9,10–seco–5,7,10(19)–cholestatriene–1,3,25,26–tetrol	473	P
31	ST03	" "	" "	1α,25–dihydroxy–11β–methoxyvitamin D3 / 1α,25–dihydroxy–11β–methoxycholecalciferol	(5Z,7E)–(1S,3R,11R)–11–methoxy–9,10–seco–5,7,10(19)–cholestatriene–1,3,25–triol	447	S
32	ST03	" "	" "	1α,25–dihydroxy–19–nor–22–oxavitamin D3 1α,25–dihydroxy–19–nor–22–oxacholecalciferol	(7E)–(1R,3R)–19–nor–22–oxa–9,10–seco–5,7–cholestadiene–1,3,25–triol	424	P / S
33	ST03	" "	" "	1α,25–dihydroxy–21–nor–20–oxavitamin D3 / 1α,25–dihydroxy–21–nor–20–oxacholecalciferol	(5Z,7E)–(1S,3R)–21–nor–20–oxa–9,10–seco–5,7,10(19)–cholestatriene–1,3,25–triol	427 / 422	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
34	ST03	"	"	1α,25–dihydroxy–26,27–dimethyl–22,22,23,23–tetradehydro–24a,24b,24c–trihomo–20–epivitamin D3 / 1α,25–dihydroxy–26,27–dimethyl–22,22,23,23–tetradehydro–24a,24b,24c–trihomo–20–epicholecalciferol	(5Z,7E)–(1S,3R,20R)–26,27–dimethyl–24a,24b,24c–trihomo–9,10–seco–5,7,10(19)–cholestatrien–22–yne–1,3,25–triol	500	P / S
35	ST03	"	"	1α,25–dihydroxy–26,27–dimethyl–22,22,23,23–tetradehydro–24a–homovitamin D3 / 1α,25–dihydroxy–26,27–dimethyl–22,22,23,23–tetradehydro–24a–homocholecalciferol	(5Z,7E)–(1S,3R)–26,27–dimethyl–24a–homo–9,10–seco–5,7,10(19)–cholestatrien–22–yne–1,3,25–triol	472	P / S
36	ST03	"	"	1α,25–dihydroxy–26,27–dimethyl–24a,24b–dihomovitamin D3 / 1α,25–dihydroxy–26,27–dimethyl–24a,24b–dihomocholecalciferol	(5Z,7E)–(1S,3R)–26,27–dimethyl–24a,24b–dihomo–9,10–seco–5,7,10(19)–cholestatrien–1,3,25–triol	490	P / S
37	ST03	"	"	1α,25–dihydroxy–6,7–didehydroprevitamin D3 / 1α,25–dihydroxy–6,7–didehydroprechole–calciferol	(1S,3R)–9,10–seco–5(10),8–cholestadien–6–yne–1,3,25–triol	447	S
38	ST03	"	"	2α–(3–Hydroxypropyl)–1α,25–dihydroxy–19–norvitamin D3	(5Z,7E)–(1R,2R,3R)–3–(hydroxy–propyl)–19–nor–9,10–seco–5,7–cholestadien–1,3,25–triol	445	P / S
39	ST03	"	"	3–Fluoro–9,10–secocholesta–5,7,10(19)–triene	(5Z,7E)–(3S)–3–fluoro–9,10–seco–5,7,10(19)–cholestatriene	404	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
40	ST03	" "	" "	ZK 168281	(5Z,7E,22E)–(1S,3R,24R)–25–(carboethoxy–methylene)–26,26–cyclo–9,10–seco–5,7,10(19),22–1,3,24–triolcholestatetraene–1,3,24–triol	528	P
41	ST04	Bile acids and derivatives	C24 bile acids, alcohols, and derivatives	–	(20S,22E)–3β–Hydroxy–5α–chol–22–en–24–oic Acid	357	P
42	ST04	" "	" "	–	(22E)–3β–Hydroxy–5α–chola–7,22–dien–24–oic Acid	373	P / S
43	ST04	" "	" "	–	12–Oxo–5β–cholan–24–oic Acid	357	P
44	ST04	" "	" "	–	2β,3β–Dihydroxy–6–oxo–5α–chol–7–en–24–oic Acid	422	P / S
45	ST04	" "	" "	–	3α–Hydroxy–1,7–dioxo–5β–cholan–24–oic Acid	422	P
46	ST04	" "	" "	–	3β,12β–Dihydroxy–5β–cholan–24–oic Acid	410	P / S
47	ST04	" "	" "	–	3β,6α,7α–Trihydroxy–5β–cholan–24–oic Acid	426	P
48	ST04	" "	" "	–	3β,9α–Dihydroxy–11–oxo–5β–cholan–24–oic Acid	424	P / S
49	ST04	" "	" "	–	3β–Hydroxy–7–oxo–5α–cholan–24–oic Acid	391	P / S
50	ST04	" "	" "	–	3β–Hydroxy–chol–5–en–24–oic Acid	357	P / S
51	ST04	Bile acids and derivatives	C27 bile acids, alcohols, and derivatives	–	5β–Cholest–25–ene–3α,7α,12α,23–tetrol	452	P
52	ST04	Bile acids and derivatives	C23 bile acids, alcohols, and derivatives	–	24–Nor–5β–chol–22–ene–3α,6α–diol	364	P / S
53	ST05	Steroid conjugates	Taurine conjugates	Taurochenodeoxycholic acid	N–(3α,7α–dihydroxy–5β–cholan–24–oyl)–taurine	517	P / S
54	ST05	" "	" "	Taurodeoxycholic acid	N–(3α,12α–dihydroxy–5β–cholan–24–oyl)–taurine	350	P
55	ST05	" "	" "	Taurohyocholic acid	N–(3α,6α,7α–Trihydroxy–5β–cholan–24–oyl)–taurine	533	S
	Glycerolipids (GLs)						
1	GL01	Monoradyl–glycerols	Monoacyl–glycerols	MG(0:0/18:1(9Z)/0:0)	2–(9Z–octadecenoyl)–sn–glycerol	374	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
2	GL01	" "	" "	MG(16:0/0:0/0:0)	1–hexadecanoyl–sn–glycerol	348 / 353	P / S
3	GL01	" "	" "	MG(17:0/0:0/0:0)[rac]	1–heptadecanoyl–rac–glycerol	362	P
4	GL01	" "	" "	MG(18:0/0:0/0:0)[rac]	1–octadecanoyl–rac–glycerol	376	P / S
5	GL01	" "	" "	MG(18:1(11E)/ 0:0/0:0)[rac]	1–(11E–octadecenoyl)–rac–glycerol	357	P
6	GL01	" "	" "	MG(18:2(9Z,12Z) /0:0/0:0)[rac]	1–(9Z,12Z–octadecadienoyl)–rac–glycerol	372	P / S
7	GL01	" "	" "	MG(20:0/0:0/0:0)[rac]	1–eicosanoyl–rac–glycerol	387	P / S
8	GL02	Diradyl–glycerols	Diacyl–glycerols	DG(12:0/17:2(9Z,12Z) /0:0)[iso2]	1–dodecanoyl–2–(9Z,12Z–heptadecadienoyl)–sn–glycerol	505	P
9	GL02	" "	" "	DG(12:0/21:0/0:0) [iso2]	1–dodecanoyl–2–heneicosanoyl–sn–glycerol	601	P / S
10	GL02	" "	" "	DG(12:0/22:2 (13Z,16Z)/0:0)[iso2]	1–dodecanoyl–2–(13Z,16Z–docosadienoyl)–sn–glycerol	576 / 611	P / S
11	GL02	" "	" "	DG(13:0/16:0/0:0) [iso2]	1–tridecanoyl–2–hexadecanoyl–sn–glycerol	544	P
12	GL02	" "	" "	DG(14:0/16:0/0:0) [iso2]	1–tetradecanoyl–2–hexadecanoyl–sn–glycerol	559	P / S
13	GL02	" "	" "	DG(14:0/20:0/0:0) [iso2]	1–tetradecanoyl–2–eicosanoyl–sn–glycerol	615	P / S
14	GL02	" "	" "	DG(14:1(9Z)/ 19:0/0:0)[iso2]	1–(9Z–tetradecenoyl)–2–nonadecanoyl–sn–glycerol	599	S
15	GL02	" "	" "	DG(14:1(9Z)/22:2 (13Z,16Z)/0:0)[iso2]	1–(9Z–tetradecenoyl)–2–(13Z,16Z–docosadienoyl)–sn–glycerol	637	P
16	GL02	" "	" "	DG(15:0/16:0/0:0) [iso2]	1–pentadecanoyl–2–hexadecanoyl–sn–glycerol	573	P / S
17	GL02	" "	" "	DG(15:0/18:1(9Z) /0:0)[iso2]	1–pentadecanoyl–2–(9Z–octadecenoyl)–sn–glycerol	582	P
18	GL02	" "	" "	DG(15:1(9Z)/19:0/ 0:0)[iso2]	1–(9Z–pentadecenoyl)–2–nonadecanoyl–sn–glycerol	613	P / S
19	GL02	" "	" "	DG(15:1(9Z)/21:0/ 0:0)[iso2]	1–(9Z–pentadecenoyl)–2–heneicosanoyl–sn–glycerol	641	P / S
20	GL02	" "	" "	DG(16:0/0:0/16:0) (d5)	1,3–dihexadecanoyl–2–hydroxy–glycerolÂ (d5)	597	P
21	GL02	" "	" "	DG(16:0/16:0/0:0) [rac]	1,2–dihexadecanoyl–rac–glycerol	587	P / S
22	GL02	" "	" "	DG(16:0/16:1(9Z)/ 0:0)[iso2]	1–hexadecanoyl–2–(9Z–hexadecenoyl)–sn–glycerol	585	P / S
23	GL02	" "	" "	DG(16:0/19:0/0:0) [iso2]	1–hexadecanoyl–2–nonadecanoyl–sn–glycerol	629	P / S
24	GL02	" "	" "	DG(16:0/22:0/0:0) [iso2]	1–hexadecanoyl–2–docosanoyl–sn–glycerol	671	P / S
25	GL02	" "	" "	DG(16:1(9Z)/0:0/ 16:1(9Z)) (d5)	1,3–di–(9Z–hexadecenoyl)–2–hydroxy–glycerol (d5)	552	S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
26	GL02	" "	" "	DG(17:0/0:0/17:0) (d5)	1,3–diheptadecanoyl–2–hydroxy–glycerolÂ (d5)	625	S
27	GL02	" "	" "	DG(17:2(9Z,12Z)/ 19:0/0:0)[iso2]	1–(9Z,12Z–heptadecadienoyl)–2–nonadecanoyl–sn–glycerol	604	P / S
28	GL02	" "	" "	DG(17:2(9Z,12Z)/ 21:0/0:0)[iso2]	1–(9Z,12Z–heptadecadienoyl)–2–heneicosanoyl–sn–glycerol	667	P
29	GL02	" "	" "	DG(18:0/18:0/0:0)	1,2–dioctadecanoyl–sn–glycerol	643	P / S
30	GL02	" "	" "	DG(18:1(9Z)/22:1 (13Z)/0:0)[iso2]	1–(9Z–octadecenoyl)–2–(13Z–docosenoyl)–sn–glycerol	700	P
31	GL02	" "	" "	DG(18:2(9Z,12Z)/ 22:0/0:0)[iso2]	1–(9Z,12Z–octadecadienoyl)–2–docosanoyl–sn–glycerol	700	S
32	GL02	" "	" "	DG(18:3(6Z,9Z,12Z)/ 18:0/0:0)[iso2]	1–(6Z,9Z,12Z–octadecatrienoyl)–2–octadecanoyl–sn–glycerol	637	S
33	GL02	" "	" "	DG(18:3(9Z,12Z,15Z) /20:2(11Z,14Z)/0:0) [iso2]	1–(9Z,12Z,15Z–octadecatrienoyl)–2–(11Z,14Z–eicosadienoyl)–sn–glycerol	661	P
34	GL02	" "	" "	DG(18:4(6Z,9Z, 12Z,15Z)/18:0/0:0) [iso2]	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–octadecanoyl–sn–glycerol	600	P / S
35	GL02	" "	" "	DG(18:4(6Z,9Z,12Z, 15Z)/20:0/0:0) [iso2]	1–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–2–eicosanoyl–sn–glycerol	663	S
36	GL03	Triradyl–glycerols	Triacyl–glycerols	TG(13:0/14:0/14:1 (9Z))[iso6]	1–tridecanoyl–2–tetradecanoyl–3–(9Z–tetradecenoyl)–sn–glycerol	894	P
37	GL03	" "	" "	TG(13:0/20:0/22:6 (4Z,7Z,10Z,13Z,16Z, 19Z))[iso6]	1–tridecanoyl–2–eicosanoyl–3–(4Z,7Z,10Z,13Z,16Z, 19Z–docosahexaenoyl)–sn–glycerol	875 / 894	P / S
38	GL03	" "	" "	TG(14:1(9Z)/18:0/ 20:2(11Z,14Z)) [iso6]	1–(9Z–tetradecenoyl)–2–octadecanoyl–3–(11Z,14Z–eicosadienoyl)–sn–glycerol	933	P
39	GL03	" "	" "	TG(14:1(9Z)/18:0/ 22:3(10Z,13Z,16Z)) [iso6]	1–(9Z–tetradecenoyl)–2–octadecanoyl–3–(10Z,13Z,16Z–docosatrienoyl)–sn–glycerol	901	S
40	GL03	" "	" "	TG(16:0/18:0/22:2 (13Z,16Z))[iso6]	1–hexadecanoyl–2–octadecanoyl–3–(13Z,16Z–docosadienoyl)–sn–glycerol	871	P
41	GL03	" "	" "	TG(16:1(9Z)/18:0/ 18:4(6Z,9Z,12Z,15Z)) [iso6]	1–(9Z–hexadecenoyl)–2–octadecanoyl–3–(6Z,9Z,12Z,15Z–octadecatetraenoyl)–sn–glycerol	942	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
42	GL03	" "	" "	TG(18:0/18:0/20:5 (5Z,8Z,11Z,14Z,17Z)) [iso3]	1,2–dioctadecanoyl–3–(5Z,8Z,11Z,14Z,17Z–eicosapentaenoyl)–sn–glycerol	1022	P
43	GL03	" "	" "	TG(18:1(9Z)/22:0/ 22:6(4Z,7Z,10Z,13Z, 16Z, 19Z))[iso6]	1–(9Z–octadecenoyl)–2–docosanoyl–3–(4Z,7Z,10Z,13Z,16Z,19Z–docosahexaenoyl)–sn–glycerol	965	P
44	GL03	" "	" "	Trilauroyl–glycerol	1,2,3–tridodecanoyl–sn–sn–glycerol	657	P / S
	Sphingolipids (SPs)						
1	SP01	Sphingoid bases	Sphingoid base homo–logs and variants	C16 Sphingosine	Hexadecasphing–4E–enine	254 / 272	P / S
2	SP01	Sphingoid bases	Sphingoid base analogs	–	2–amino–14,16–dimethyloctadecan–3–ol	336	S
3	SP01	" "	" "	(4E,8E,10E–d18:3) sphingosine	sphinga–4E,8E,10E–trienine	296 / 278	P / S
4	SP01	" "	" "	(4E,8E,9Me–d19:2) sphingosine	9–methyl–sphinga–4E,8E–dienine	294	S
5	SP01	" "	" "	(4E,8E,d18:2) sphin-gosine	sphinga–4E,8E–dienine	280	S
6	SP01	" "	" "	6–hydroxysphingosine	6–hydroxysphing–4E–enine	338	P
7	SP01	" "	" "	Clavepictine B	(9aS)–6S–(deca–1,3E–dienyl)–4S–methyloctahydro–1H–quinolizin–3R–ol	288	S
8	SP01	" "	" "	Lepadin D	5S–(5S–hydroxyoctyl)–2S–methyldecahydroquinolin–3R–ol	280	P
9	SP01	" "	" "	N,N–dimethyl–Safingol	2S–dimethylaminooctadecane–1,3R–diol	330	S
10	SP01	" "	" "	Obscuraminol A	1–deoxy–sphinga–6Z,9Z,12Z,15Z–tetraenine	278	P / S
11	SP01	" "	" "	Prosopinine	6R–(8–hydroxydecyl)–2R–(hydroxymethyl)–piperidin–3R–ol	288	S
12	SP02	Ceramides	N–acyl–sphingo–sines (cera–mides)	Cer(d18:1/17:0)	N–(heptadecanoyl)–sphing–4–enine	585	P / S
13	SP02	" "	" "	Cer(d18:1/18:0)	N–(octadecanoyl)–sphing–4–enine	567	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
14	SP02	" "	" "	Cer(d18:1/22:0)	N–(docosanoyl)–sphing–4–enine	623	P / S
15	SP02	" "	" "	Cer(d18:1/23:0)	N–(tricosanoyl)–sphing–4–enine	637	P / S
16	SP02	" "	" "	Cer(d18:1/24:0)	N–(tetracosanoyl)–sphing–4–enine	651	P / S
17	SP02	" "	" "	Cer(d18:1/24:1(15Z))	N–(15Z–tetracosenoyl)–sphing–4–enine	649	P / S
18	SP02	" "	" "	Cer(d18:1/25:0)	N–(pentacosanoyl)–sphing–4–enine	665	P / S
19	SP02	" "	" "	Cer(d18:2/23:0)	N–(tricosanoyl)–4E,14Z–sphingadienine	635	P / S
20	SP02	Ceramides	N–acyl–sphinganines (dihydroceramides)	Cer(d18:0/14:0)	N–(tetradecanoyl)–sphinganine	513	S
21	SP02	Ceramides	N–acyl–4–hydroxy–sphinganines (phytoceramides)	Cer(t18:0/24:0(2OH))	N–(2–hydroxytetracosanoyl)–4R–hydroxy– sphinganine	723 / 685	P / S
22	SP02	Ceramides	Ceramide 1–phosphates	CerP(d18:1/24:0)	N–(tetracosanoyl)–sphing–4–enine–1–phosphate	763	S
23	SP03	Phospho–sphingolipids	Ceramide phosphocholines (sphingomyelins)	SM(d18:2/24:1)	N–(15Z–tetracosenoyl)–4E,14Z–sphingadienine–1–phosphocholine	812	P / S
24	SP03	" "	" "	SM(d16:1/18:1)	N–(9Z–octadecenoyl)–hexadecasphing–4–enine–1–phosphocholine	702	P / S
25	SP03	" "	" "	SM(d16:1/22:0)	N–(docosanoyl)–hexadecasphing–4–enine–1–phosphocholine	782	P
26	SP03	" "	" "	SM(d18:0/16:0)	N–(hexadecanoyl)–sphinganine–1–phosphocholine	706	P
27	SP03	" "	" "	SM(d18:2/18:0)	N–(octadecanoyl)–4E,14Z–sphingadienine–1–phosphocholine	730	P / S
28	SP03	" "	" "	SM(d16:1/18:0)	N–(octadecanoyl)–hexadecasphing–4–enine–1–phosphocholine	704	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
29	SP03	" "	" "	SM(d18:1/18:0)	N–(octadecanoyl)–sphing–4–enine–1–phosphocholine	732	P / S
30	SP03	" "	" "	SM(d18:1/24:0)	N–(tetracosanoyl)–sphing–4–enine–1–phosphocholine	816	P / S
31	SP03	" "	" "	SM(d18:1/23:0)	N–(tricosanoyl)–sphing–4–enine–1–phosphocholine	802	S
32	SP05	Neutral glycosphingolipids	GalNAcβ1–3Galα1–4Galβ1–4Glc– (Globo series)		Galα1–4Galβ1–4Glcβ–Cer(d18:1/24:0)	975	P / S
33	SP05	Neutral glycosphingolipids		Etn–1–P–Cer(d14:1/18:0)		690	P
34	SP05	" "		GalCer(d18:1/16:0)		718	P / S
35	SP05	" "		GalCer(d18:1/20:0)		779	P
36	SP06	Acidic glycosphingolipids	Gangliosides	–	Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ–Cer(d18:1/16:0)	1154	P / S
37	SP06	" "	" "	–	Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ–Cer(d18:1/18:0)	1182	P / S
38	SP06	Acidic glycosphingolipids	Sulfoglycosphingolipids (sulfatides)	(3'–sulfo)Galβ–Cer(d18:1/16:0)	N–hexadecanoyl–1–β–(3'–sulfo)–glucosyl–sphing–4–enine	803	P
39	SP06	Acidic glycosphingolipids	Phosphoglycosphingolipids	GlcNα1–6Ins–1–P–Cer(t18:0/26:0)	N–(hexacosanoyl)–4R–hydroxysphinganine–1–phospho–(1'–[2–amino–2–deoxy–D–glucopyranosyl–α1–6–D–myo–inositol])	971	S
	Prenol Lipids (PRs)						
1	PR01	Isoprenoids	C10 isoprenoids (monoterpenes)	(+)–trans–allethrin	2–methyl–4–oxo–3–(prop–2–en–1–yl)cyclopent–2–en–1–yl (1R,3R)–2,2–dimethyl–3–(2–methylprop–1–en–1–yl)cyclopropanecarboxylate	285	P
2	PR01	" "	" "	(+)–β–Phellandrene	–	159	P
3	PR01	" "	" "	(4R,6R)–cis–Carveol	–	153	P / S
4	PR01	" "	" "	Iridotrial	–	183	S
5	PR01	" "	" "	β–Dolabrin	–	180	P

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
6	PR01	Isoprenoids	C15 isoprenoids (sesquiterpenes)	(–)–Solavetivone	–	219	P
7	PR01	" "	" "	(+)–Blennin D	–	284 / 267	P / S
8	PR01	" "	" "	5–(1–hydroxybutan–2–yl)isolongifol–4–ene	–	309	S
9	PR01	" "	" "	5–(2–methoxyethyl)isolongifol–5–ene	(1R,8S)–5–(2–methoxyethyl)–2,2,7,7–tetramethyltricyclo[6.2.1.0(1,6)]undec–5–ene	263 / 280	P / S
10	PR01	" "	" "	Acutifolane A	–	295	S
11	PR01	" "	" "	alantolactone	(3aR,5S,8aR,9aR)–5,8a–dimethyl–3–methylidene–3a,5,6,7,8,8a,9,9a–octahydronaphtho[2,3–b]furan–2(3H)–one	265	P
12	PR01	" "	" "	Botrydial	–	293	P
13	PR01	" "	" "	Dendrobane A	–	253	S
14	PR01	" "	" "	Emmotin A	–	279	P / S
15	PR01	" "	" "	Juvenile hormone III	–	249 / 284	P / S
16	PR01	" "	" "	Latia luciferin	(1E)–2–methyl–4–(2,6,6–trimethylcyclohex–1–en–1–yl)but–1–enylÂ formate	237	P
17	PR01	" "	" "	Sugeonol	8S–hydroxy–1,4R,9,9–tetramethyl–3,4,5,6,7,8–hexahydro–3a(R),7–methanoazulen–2–one	235	P / S
18	PR01	" "	" "	Sugeonyl acetate	8S–acetoxy–1,4R,9,9–tetramethyl–3,4,5,6,7,8–hexahydro–3a(R),7–methanoazulen–2–one	294	P / S
19	PR01	Isoprenoids	C20 isoprenoids (diterpenes)	(–)–Asbestinine 2	–	494	P
20	PR01	" "	" "	Eremolactone	–	321 / 316	P / S
21	PR01	" "	" "	gibberellin A17	–	396	P
22	PR01	" "	" "	Neoabietic acid	–	303	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
23	PR01	Isoprenoids	C25 isoprenoids (sesterterpenes)	(–)–Ceriferol 1	–	374	P
24	PR01	Isoprenoids	C30 isoprenoids (triterpenes)	gypsogenin	3β–hydroxy–23–oxoolean–12–en–28–oic acid	488	P
25	PR01	Isoprenoids	C40 isoprenoids (tetraterpenes)	4,4'–Diapolycopene	–	383	P / S
26	PR01	" "	" "	4,4'–Diapophytoene/ Dehydrosqualene	–	409	S
27	PR01	" "	" "	4'–Apo–3,4–didehydrolycopene/ (4–Apo–3',4'–didehydrolycopene)	–	484	P
28	PR01	" "	" "	7,8–Didehydroastaxanthin	–	612	P
29	PR01	" "	" "	Spheroidene	–	587	S
30	PR01	Isoprenoids	Retinoids	11–cis–retinal	–	302	S
31	PR01	" "	" "	13–cis–retinoic acid, Isotretinoin	–	318 / 301	P / S
32	PR01	" "	" "	19–Oxo–9–cis–retinoic acid	–	315	P
33	PR01	" "	" "	9–cis–retinol	9Z–retinol	304	P / S
34	PR01	" "	" "	All–Trans–3,4–Didehydro–Retinoic acid	–	316	S
35	PR02	Quinones and hydroquinones	Ubiquinones	Plastoquinol–1	2,3–dimethyl–5–(3–methylbut–2–enyl)–benzene–1,4–diol	362 / 345	P / S
36	PR02	Quinones and hydroquinones	Vitamin E	alhpa–tocopheronolactone	–	279	P / S
37	PR04	Hopanoids	–	Diploptene	Hop–22(29)–ene	428	S
	Polyketides (PKs)						
1	PK01	Linear polyketides	–	Trichostatin	–	325	S
2	PK04	Macrolides and lactone polyketides	–	6–deoxyerythronolide B	–	387 / 404	P / S
3	PK04	" "	–	Zearalenone	–	319	P / S

Table A.1: Complete canine plasma (P) and serum (S) lipidome using untargeted LC/MS, no internal standards, lipid extraction by Matyash et al. (2008) and lipid identification by LipidMaps.

Nr.	Main Cat.	Main Class	Sub Class	Common Name	Systematic Name	m/z measured	Plasma (P) / Serum (S)
4	PK12	Flavonoids	Isoflavans	Machaerol C	6,8,2'–Trihydroxy–7,3',4'–trimethoxyisoflavan	331	P / S
5	PK12	Flavonoids	Chalcones and dihydrochalcones	–	1–(2,5–dihydro–6,8–dihydroxy–3–methyl–1–benzoxepin–7–yl)–3–phenyl–1–propanone	325	P / S
6	PK12	" "	" "	Crotaoprostrin	2'–Hydroxy–3,4,5–methoxychalcone	332	P
7	PK12	" "	" "	Desmosdumotin C	–	313	P / S
8	PK12	" "	" "	Pongagallone A	–	353	P
9	PK12	" "	" "	Prorepensin	5,3'–Digeranyl–3,4,2',4'–tetrahydroxychalcone	562	P / S
10	PK12	" "	" "	Syzygiol	–	517 / 332	P / S
11	PK12	Flavonoids	Flavanones	Brosimacutin C	–	360	P / S
12	PK13	Aromatic polyketides	Diphenyl ethers, biphenyls, dibenzyls and stilbenes	Glepidotin C	–	331	P
	Saccharolipids (SLs)						
1	SL05	Other acyl sugars	–	–	6–O–(3R,4–dihydroxy–2–methylene–butanoyl)–β–D–glucopyranose	312	S

A.2 Plasma vs. Serum: QQ–Plots

Plasma

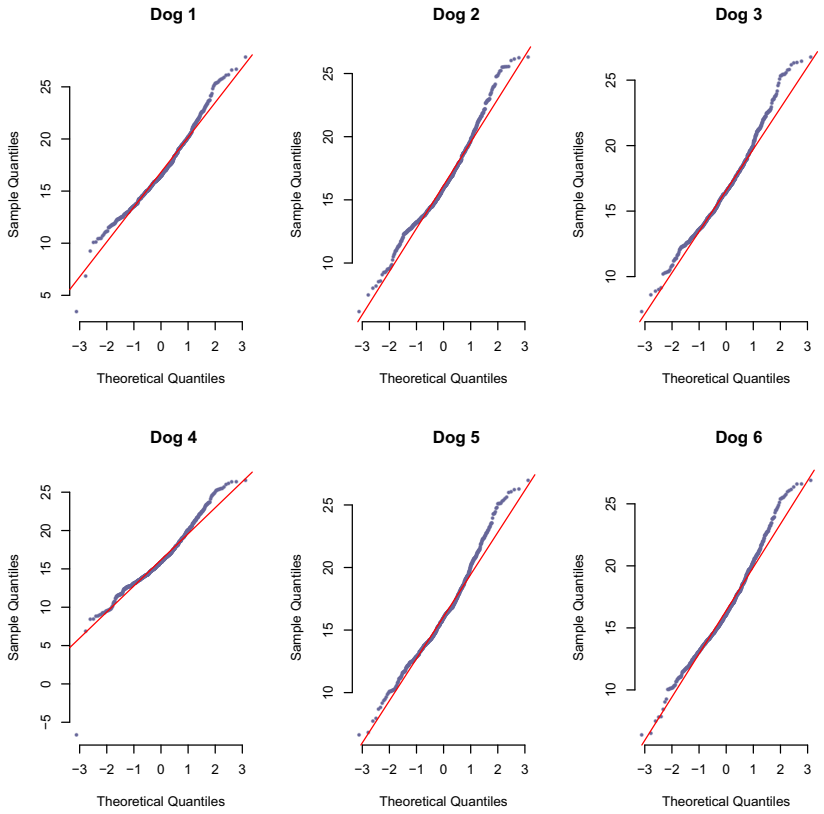

Figure A.1: QQ–Plots of canine lithium heparin plasma before treatment with prednisolone, indicating normal distribution of lipids (for biological samples) within the six dogs.

Serum

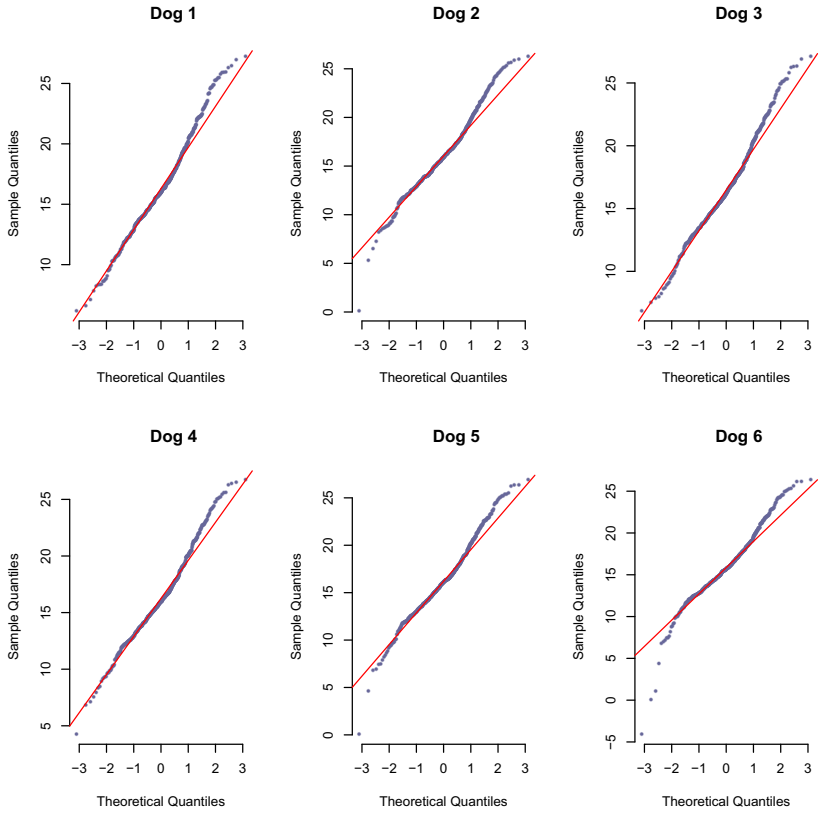

Figure A.2: QQ–Plots of canine serum before treatment with prednisolone, indicating normal distribution of lipids (for biological samples) within the six dogs.

A.3 Prednisolone Treatment: QQ-Plots

Six healthy Beagles were treated with prednisolone and blood was taken before (A) and after (B) treatment. Using logarithmic transformation, results were presumed to be normally distributed regarding slight biological variations.

Plasma

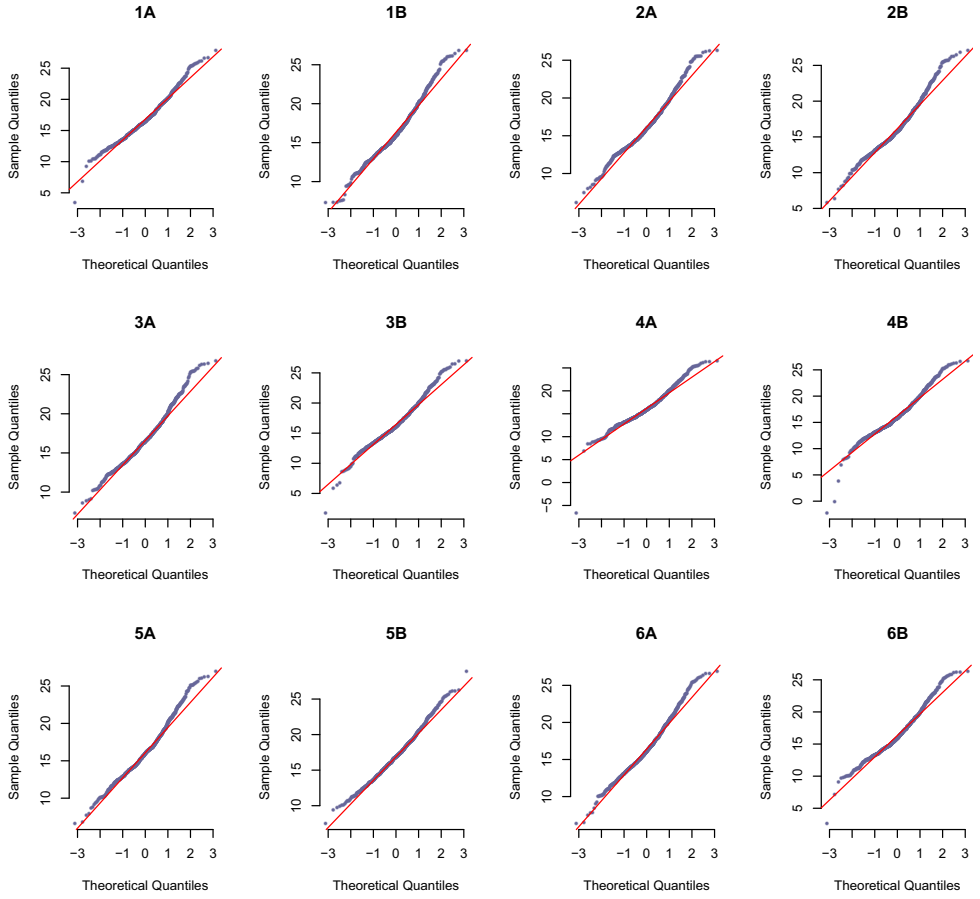

Figure A.3: QQ–Plot using plasma from dog 1–6. A = before treatment. B = after treatment. Results can be presumed as normally distributed after log 2 transformation.

Serum

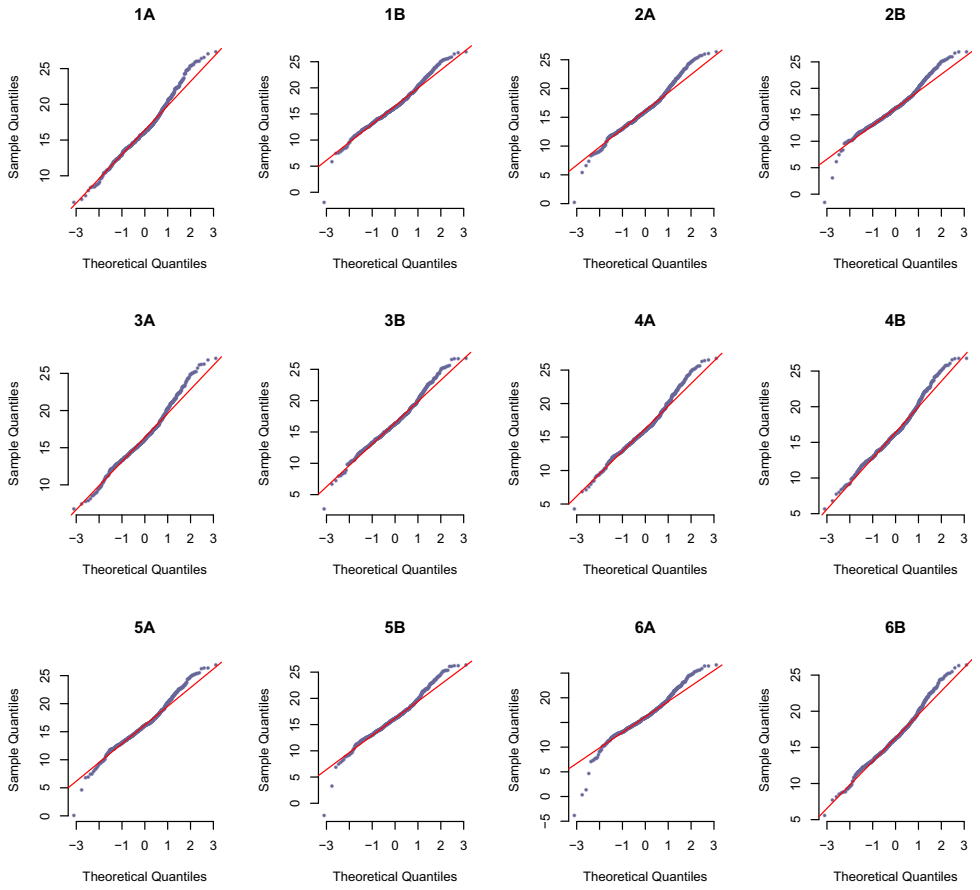

Figure A.4: QQ–Plot using serum from dog 1–6. A = before treatment. B = after treatment. Results can be presumed as normally distributed after log 2 transformation.